Studies on the Pathophysiological Aspects of the Metabolic Syndrome in Transgenic Mice

Studies on the Pathophysiological Aspects of the Metabolic Syndrome in Transgenic Mice

Proefschrift

ter verkrijging van

de graad van Doctor aan de Universiteit van Leiden

op gezag van Rector Magnificus prof. mr. P.F. van der Heijden

volgens besluit van het College voor Promoties

te verdedigen op woensdag 25 februari 2009

klokke 13:45 uur

door

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geboren te Yu Hu, Zhejiang Province (China)

in 1976

Promotiecommissie

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The studies presented in this thesis were performed at the Gaubius Laboratory of TNO-Quality of Life and the department of General Internal Medicine/Endocrinology and Metabolic Diseases at the Leiden University Medical Center, Leiden, The Netherlands.

The research was partly financially supported by a grand from The Netherlands Organisation of Scientific Research Mosaic (NWO 017.003.083).
Financial support by The Netherlands Heart Foundation and J.E. Juriaanse Stichting for the publication of this thesis is gratefully acknowledged.

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Cover photo: Urban Display
Cover design: Textcetera
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Printing: Ipskamp Drukkers B.V.

The printing of this thesis was financially supported by:
Institute for cardiovascular research VUMC and Leiden Vascular Medicine
International Atherosclerosis Society
Arie Blok Diervoeding
AstraZeneca B.V.
Boehringer Ingelheim
Eli Lilly Nederland B.V.
Genzyme Nederland
GlaxoSmithKline
Menarini Farma Nederland
Merck Sharp & Dohme
NovoNordisk
Schering-Plough B.V.
Zambom

ISBN: 978 90 79969 02 9
NUR: 871

Lihui Hu
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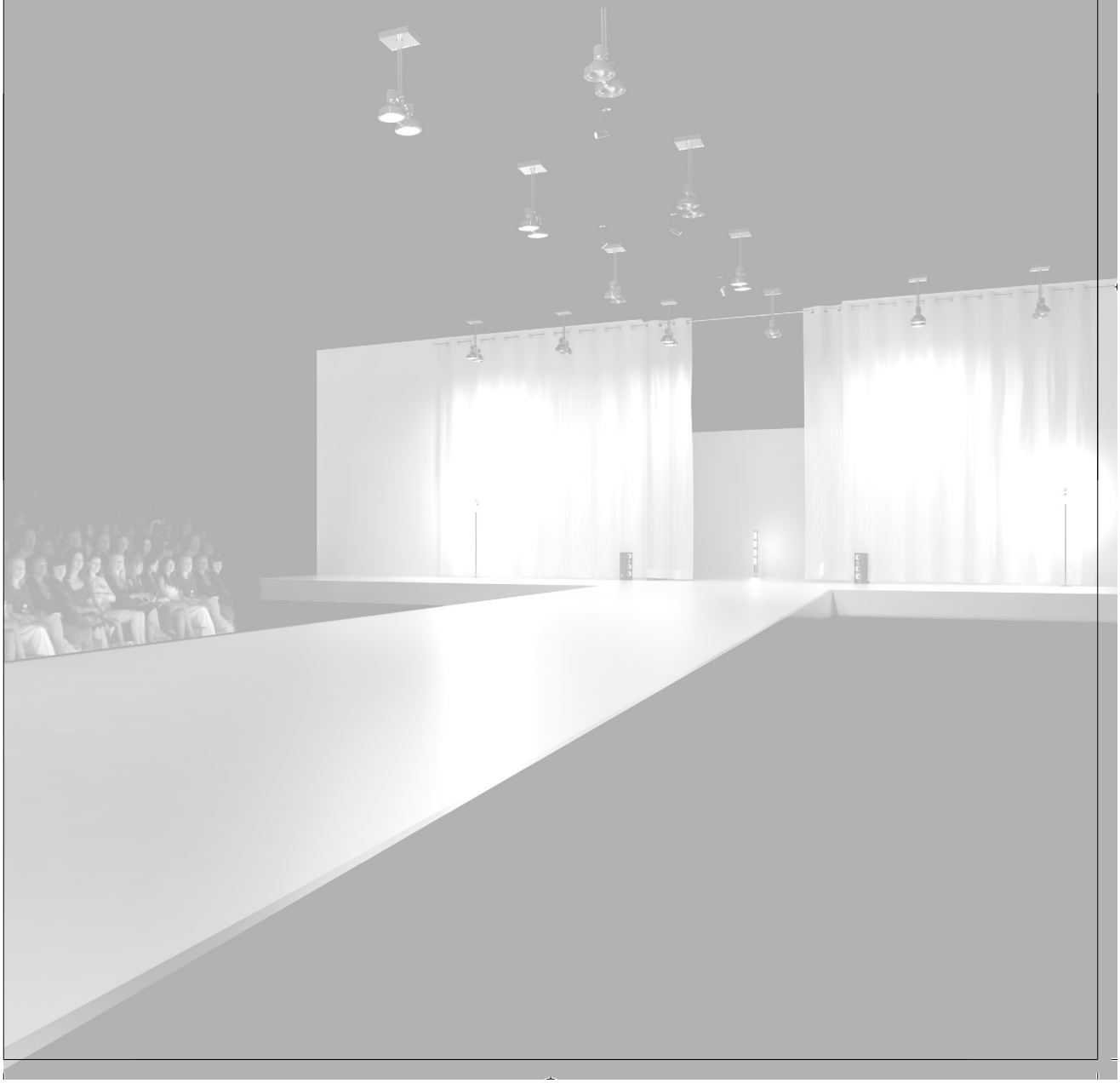
Proefschrift Leiden
Met literatuuropgave – Met samenvatting in het Nederlands

Contents

Chapter 1

General Introduction



1.1 Metabolic syndrome

1.1.1 Definition

Overweight and obesity is rapidly becoming a major health problem, momentarily affecting more than 1 billion adults, and over 300 million of them clinically obese. It is predicted by the World Health Organization (WHO) that by 2015, approximately 2.3 billion adults will be overweight and more than 700 million will be obese.[1] This overweight/obesity pandemic is not only restricted to adults, but childhood obesity is already epidemic in some regions. It is estimated that around 10% of the youths are obese worldwide. Overweight and obesity lead to adverse metabolic effects on blood pressure, lipid metabolism and insulin resistance. This clustering of pathologies is called as the metabolic syndrome (MetS) and has also started to emerge in children at young ages, a phenomenon that was inconceivable a few decades ago.

The MetS is also known syndrome X, Reaven's syndrome and insulin resistance syndrome. The latter and MetS are now commonly and interchangeably used names. MetS was first described in early 1920s by Kylin as a constellation of hypertension, hyperglycaemia and gout.[2] Over the years, different criteria were used to define the MetS. In late 1940s the MetS was redefined by Vague in which android or male-type obesity was included.[3] In 1988 Reaven stated the clinical importance of the MetS. In the landmark publication of his 1988 Banting Medal award lecture, Reaven described syndrome X as a constellation of insulin resistance, hyperglycaemia, hypertension, low high-density lipoprotein cholesterol (HDL-C) levels and increased very low-density lipoprotein (VLDL)-triglyceride levels.[4] In 1999, World Health organisation (WHO) has attempted to create an international unifying guideline.[5] However, the numbers of metabolic disorders that are associated with the MetS has increased in the last few years. Therefore, different expert groups (the European Group for the Study of Insulin Resistance (EGIR), the International Diabetes Federation (IDF) and the Third Report of the National Cholesterol Education Program's Adult Treatment Panel (NCEP ATP III) redefined the MetS and modified the WHO definition (**Table 1**).[6-9] Nowadays, the WHO and NCEP ATP III definitions are most commonly used. The exact pathogenesis of the MetS is not clear. It is suggested that MetS is the result of increasingly sedentary lifestyles combined with ready access to energy-rich food sources in genetically susceptible individuals. Subjects with the MetS have high risk for developing insulin resistance and cardiovascular diseases.

Table 1 **The different definitions of the Metabolic Syndrome**

	Major criteria	Minor criteria				
WHO	type II diabetes mellitus or impaired fasting glucose or insulin resistance	obesity BMI > 30 kg/m^2 and/or waist/hip ratio >0.9 (♂) or >0.85 (♀)	dylipidemia HDL-C < 0.9 (♂) or < 1.0 mM ♀ TG ≥ 1.7 mM	hypertension BP > 140/90 mm Hg	microalbuminuria urinary albumin excretion rate > 20 µg/min or albumin/ creatinine ratio ≥ 30 mg/g	
EGIR	insulin resistance or fasting hyper-insulinaemia	central obesity waist circumference 94 cm (♂) 80 cm (♀)	dyslipidemia HDL-C < 1.0 mM TG > 2.0 mM	hypertension BP ≥ 140/90 mm Hg	FPG ≥ 6.1 mM	
NCEP ATP III		central obesity Waist circumference 102 cm (♂) or 88 cm (♀)	low HDL-C < 1.03 mM (♂) < 1.29 mM (♀)	triglycerides ≥ 1.7 mM	Hypertension BP ≥ 135/80 mm Hg	FPG ≥ 6.1 mM
IDF	Central obesity	low HDL-C < 1.03 mM (♂) < 1.29 mM (♀) or treatment for this abnormality	triglycerides ≥ 1.7 mM or treatment of this abnormality	hypertension systolic BP ≥130 mm Hg, diastolic BP > 85 mM Hg or treatment for previously diagnosed hypertension	FPG ≥ 5.6 mM	

HDL-C: high density lipoprotein-cholesterol
TG: triglyceride
BP: blood pressure
FPG: fasting plasma glucoses
EGIR: European Group for the Study of Insulin Resistance, IDF: International Diabetes Federation, NCEP ATP III: Third Report of the National Cholesterol Education Program's Adult Treatment Panel, HDL-C: high density lipoprotein-cholesterol, TG: triglyceride, BP: blood pressure, FPG: fasting plasma glucoses.

1.1.2 Prevalence and clinical consequences

The worldwide prevalence of MetS is not exactly known, because of different definitions are being used. Therefore comparisons in the prevalence are difficult to make. However, most studies do agree that the prevalence is rapidly increasing. Particular alarming is the increase in children. Consistent observations are that the prevalence is age-dependent and with a high ethnic variation. In the United States almost 25% of the total population has the MetS according to the NCEP ATP III definition.[10] In Europe the prevalence of the MetS varies between 24.5% in Greece and 32.6% in Spain.[11,12] In Asia, the prevalence is less than 20%.[13,14]

The most important clinical consequences of MetS are the insulin resistance and the cardiovascular diseases. MetS increases the risk for type 2 diabetes mellitus independent of insulin resistance.[15] Moreover, the MetS is associated with an increased risk for cardiovas-

cular mortality and morbidity.[16-18] Subjects with MetS have 3 times higher risk for dying of cardiovascular heart diseases when compared with subjects without MetS. MetS is also associated with chronic kidney diseases independent of the presence of type 2 diabetes mellitus or hypertension, although the underlying mechanism is not known.[19,20] Simultaneous occurrence of both metabolic syndrome and insulin resistance worsens the risk for cardiovascular diseases. Taken together, the MetS has major clinical impacts.

1.1.3 Insulin resistance

Under normal physiological conditions, ingested glucose is taken up and stored in the liver and in insulin-sensitive peripheral tissues (predominantly the skeletal muscle and the adipose tissue). Plasma glucose stimulates the production and secretion of insulin by the pancreatic insulin producing β-cells. On the one hand, insulin inhibits hepatic glucose production by inhibiting the glycogenolysis and gluconeogenesis. On the other, insulin stimulates the uptake of glucose and the formation of glycogen in the skeletal muscle. Furthermore, insulin inhibits the production of lipoprotein particles and energy substrates such as lactate and free fatty acids (FFAs) in the liver and the adipose tissue. In the adipose tissue, insulin also promotes the synthesis of triglycerides (TG) as energy storage and inhibits lipolysis. In obese state, energy intake exceeds the capacity to store energy in the adipose tissue leading to energy 'overflow' to ectopic sites. These ectopic sites are observed in the liver, skeletal muscle and pancreatic insulin-secreting β-cells.[21,22] The liver plays a pivotal role in maintaining the glucose and lipid metabolism. Several clinical studies have shown that lipid accumulation in the liver is associated with insulin resistance.[23] Patients with type 2 diabetes mellitus have a defect glucose metabolism in the liver and skeletal muscles.[24,25]

Under insulin resistance conditions, insulin fails to suppress the production of glucose and energy substrates, to stimulate the glucose uptake, and to inhibit lipolysis. Therefore, Insulin resistance is defined as a state of reduced responsiveness to normal circulating insulin levels affecting multiple organs.
Insulin resistance is a key component in the pathogenesis of the metabolic syndrome and type 2 diabetes mellitus. Numerous studies have shown that obesity and overweight are the major contributors of the metabolic syndrome. It is agreed that impaired glucose metabolism, exacerbate lipid accumulation and inflammatory processes contribute to insulin resistance. The pathogenesis of insulin resistance has been studied extensively; much is still to do since the incidence of insulin resistance has become epidemic.

1.2 PAI-1 and insulin resistance

Several epidemiological studies have shown association between increased plasma PAI-1 levels and body mass index, triglyceride levels and insulin resistance.[26-29] In the Insulin Resistance Atherosclerosis Study (IRAS) plasma PAI-1 levels predict the development of diabetes independently from other known risk factors.[26] Progression of PAI-1 plasma levels in addition to initial high plasma levels are associated with the incident diabetes.[30] Improving insulin resistance by diet, exercise or oral antidiabetic drug treatment results in decreased plasma levels of PAI-1 antigen and activity.[31-34] Although, PAI-1 is known to be synthesized by various tissues including liver and adipose tissue, the source and the mechanism of increased plasma PAI-1 levels in obesity and insulin resistance are incompletely understood. Increased plasma PAI-1 in obesity might be derived from the adipose tissue. Alternatively, increased plasma PAI-1 can be the result of local and systemic production following stimulation by adipokines.

The expression of PAI-1 in adipose tissues is positively correlated with obesity in human and rodents, suggesting a possible role in the development of obesity and insulin resistance.[35-39] This is supported by the observation made in genetically obese and insulin resistant mouse models. Disruption of the *PAI-1 gene* in ob/ob mice reduces adiposity and improves the metabolic profile determined by glucose and insulin tolerance test.[40] Two other studies showed that mice lacking PAI-1 do not develop diet-induced obesity and insulin resistance.[41,42] Downregulation of PAI-1 by angiotensin type I receptor antagonist in wild-type (WT) mice ameliorates diet-induced obesity, hyperglycemia and hyperinsulinemia. Administration of synthetic PAI-1 inhibitor induces higher insulin sensitivity in WT mice.[43]These studies suggest that PAI-1 may not merely increase in response to obesity and insulin resistance, but may have direct causal role in the development of obesity and insulin resistance. In contrast to these studies, PAI-1 deficient mice kept on a high fat diet for 3-8 weeks develop more adipose tissue.[44] In agreement with this, transgenic mice overexpressing PAI-1 have lower body weight, lower adipose tissue mass and less intraperitoneal fat.[45]
Taken together, although strong clinical evidence is present that PAI-1 plays an important role in insulin resistance and obesity, it is not clearly confirmed yet by experimental studies how enhanced PAI-1 is linked to the pathological conditions of insulin resistance and obesity. Does PAI-1 contribute to the pathogenesis of insulin resistance and obesity? Or is PAI-1 merely an epiphenomenon of insulin resistance and obesity?

1.3 Atherosclerosis

Cardiovascular disease (CVD) includes myocardial infarction, congestive heart failure, stroke and peripheral artery diseases. CVD is the leading cause of all mortality and morbidity worldwide. In North America more than 1 out the 3 persons will die of CVD and it is predicted that health costs will exceed 430 billion dollars.[46] Comparable mortality numbers hold true for The Netherlands. Atherosclerosis is the primary cause of CVD. Atherosclerosis is a progressive disease of the vessel wall that already begins in young adults. The disease primarily occurs in the large and medium-sized elastic and muscular arteries. The aetiology is very complex and it involves genetic, environmental factors and the interaction between these factors. Among the risk factors are diabetes mellitus, dyslipidemia, smoking, hypertension, gender, age and physical activity. Thus, atherosclerosis results from the combination of genetic susceptibility and unhealthy environmental influences.

1.3.1 Pathogenesis of atherosclerosis

Although the knowledge of atherosclerosis has expanded in the last decades, the exact mechanism underlying the pathogenesis is still not fully understood. The traditional view of the pathogenesis of atherosclerosis is the imbalance between cholesterol deposition and removal in the subendothelial layer after injury to the endothelium.[47] The accumulation of cholesterol can be facilitated by increased plasma LDL cholesterol levels leading to proliferation of smooth muscle cells (SMC). In the subendothelial layer, LDL cholesterol can be modified and subsequently engorged by resident macrophages to form foam cells (**Figure 1**). These foam cells form the initial fatty streak lesions which precede the formation of complex fibrous lesions.

The current concept involves inflammation and atherosclerosis is now also considered as an inflammatory disease of the large and medium-sized arteries. Inflammatory processes are present in all stages of atherosclerosis progression (**Figure 1**). Triggers of atherosclerosis, such as modified LDL can stimulate endothelial cells to produce an array of inflammatory proteins including chemotactic factors like monocyte chemoattractant protein-1 (MCP-1), growth factors such as macrophage colony-stimulating factor (M-CSF) and adhesion molecules. Among the adhesion molecules are vascular cell adhesion molecule-1 (VCAM-1), intracellular cell adhesion molecule-1 (ICAM), P-selectin and E-selectin. These adhesion molecules and chemotactic factors attract monocytes and T cells into the subendothelial layer initiating the formation of the early atherosclerotic plaque. The proliferation and differentiation of the attracted monocytes are then stimulated by M-CSF. These attracted monocytes and T cells on their turn can release inflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) that further amplify the inflam-

matory activity in the vessel wall. As the atherosclerotic lesion progresses, macrophages and T cells stimulate the migration of smooth muscle cells (SMC) into the intima and the production of collagen. A fibrous cap is then formed together with extracellular lipid deposits, SMC-derived extracelluar matrix, and often with necrosis. A complex atherosclerotic lesion is then a fact. Such a complex lesion can rupture depending on the composition and vulnerability. Vulnerable plaques usually have thin fibrous caps and increased number of inflammatory cells. The fibrous cap reflects the balance between matrix production by SMC and degradation by matrix metalloproteinases. Calcification and neovascularisation can also influence the stability of the atherosclerotic plaque. In addition, thrombogenicity of a lesion depends on the presence of proteins of the coagulation cascade such as tissue factor and plasminogen activators. Usually a plaque ruptures at the edges of the lesion leading to thrombus formation and occlusion of the artery and subsequently a cardiovascular event.

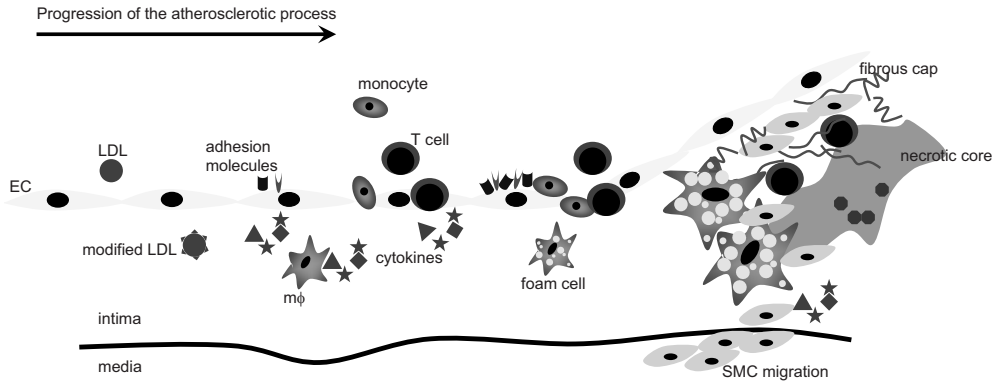


Figure 1 **Atherosclerotic process**
In the early atherosclerotic process monocytes adhere, migrate, take up modified LDL and differentiate into macrophage foam cells. The macrophage foam cells produce and release cytokines attracting even more inflammatory cells, such as T cells. In the advance process, smooth muscle cells migrate and proliferate to form fibrous cap overlying a poll of lipid-laden macrophages, T cells, necrosis, and cholesterol crystals. EC: endothelial cells, LDL: low-density lipoprotein, SMC: smooth muscle cells

1.3.2 Inflammation and atherosclerosis

As discussed above (**section pathogenesis of atherosclerosis**) inflammatory processes play a key role in the development of atherosclerosis. The nuclear factor κB (NF-κB) is a central regulatory factor of the inflammatory processes. NF-κB is considered to play a crucial role atherosclerosis locally at the vessel wall. Many inducers and target genes of NF-κB are implicated to be involved throughout the atherosclerotic process.[48] In the initial phase NF-κB can be activated in the endothelium by atherosclerogenetic stimuli such as modified LDL and

inflammatory cytokines produced at the lesion site. Additionally, NF-κB is demonstrated to be involved in the regulation of the modification of LDL, the expression chemokines and adhesion molecules.[49-56] All are important in the initial phase of the atherosclerotic process. In the advanced lesions NF-κB plays an important role in SMC migration and proliferation. The stability of an atherosclerotic plaque may also be governed by NF-κB by controlling apoptosis and necrosis. Macrophage-specific deletion of the main NF-κB activator IKK2 results in atherosclerotic lesions with increased necrosis and apoptosis.[57] However, reduced activity of NF-κB not only results in increased cell death, but also in reduced secretion of the anti-inflammatory cytokine IL-10. In the same setting reduced secretion of the pro-inflammatory cytokine TNF-α is also observed. In line with these findings, mice with p50 deficiency in the hematopoietic system show reduce less atherosclerosis, but more inflammation in the lesions.[58] Thus, this emphasizes that NF-κB as the central regulatory factor of inflammation has a complex role by influencing both pro-atherogenic and anti-atherogenic process in the vessel wall. Therefore, much is still to do to disentangle how the NF-κB activation and signalling pathways are orchestrated during the development of atherosclerosis.

In contrast, not many studies have been performed to investigate the underlying mechanisms of systemic inflammation on the development of atherosclerosis. Countless epidemiologic studies have shown that low-grade systemic inflammation is associated with metabolic syndrome. The liver is the key regulatory organ in the systemic inflammatory processes. The production of acute phase proteins, like C-reactive protein (CRP), serum amyloid A (SAA), plasminogen activator inhibitor-1 (PAI-1) are most relevant in this respect. CRP and PAI-1 are increased in subjects with the metabolic syndrome. PAI-1 has been shown to increase the risk of atherothrombotic events and may also promote the progression of atherosclerosis.[59] Experimental studies have demonstrated that CRP can activate endothelial cells to produce inflammatory markers. Furthermore, SAA can stimulate the cholesterol uptake by smooth muscle cells in an atherosclerotic plaque.[60] Therefore, hepatic inflammatory parameters are considered to be strongly associated with atherosclerosis and cardiovascular diseases. However, the exact mechanism by with systemic inflammation affects the development of atherosclerosis at the vessel wall has not been identified.

1.3.3 Endothelial progenitor cells and atherosclerosis

The first manifestation of atherosclerosis is the development of endothelial dysfunction, which is characterized by an activation of endothelial cells (EC) and decreased nitric oxide availability and deterioration of the endothelial monolayer. The initial damage is reversible. However, when no sufficient repair mechanism is present, ongoing deterioration of the endothelial monolayer can lead to the development of atherosclerotic lesions. The underlying molecular mechanism of endothelial repair is not fully understood.

A population of pluripotent cells within the peripheral blood has been described that are capable to differentiate into endothelial cells.[61] These endothelial progenitor cells (EPC) are able to home to sites of injury in the vascular endothelium and subsequently enhance neoangiogenesis after tissue ischemia. Therefore, the concept rose that EPC are recruited from the bone marrow to sites of damaged endothelium, where they can home and differentiated into mature endothelium cells (**Figure 2**). The phenotypic and functional characteristics of EPC are divergent. The widely accepted consensus defines cells positive for surface markers CD34 and vascular endothelial growth factor receptor-1 (VEGFR-2) as EPC.

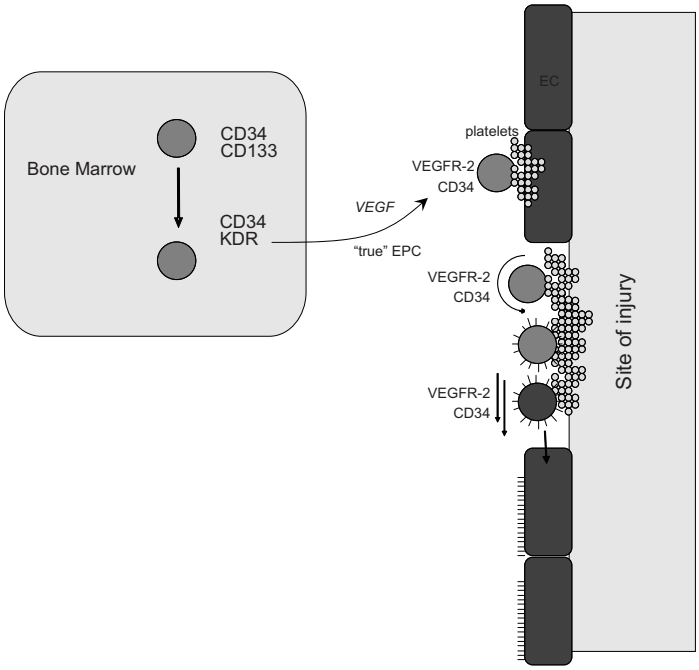


Figure 2 **Recruitment, homing and differentiation of an endothelial progenitor cell**
The endothelial progenitor cell recruited via vascular endothelial growth factor and homes to the site of injury where it differentiates into an endothelial cell. There it forms a new endothelial layer. EPC: endothelial progenitor cell, VEGF: vascular endothelial growth factor, VEGFR-2: vascular endothelial growth factor receptor-2. (courtesy of prof. dr. A.J. van Zonneveld)

The number and the functional activity of circulating EPC are correlated with cardiovascular risks. The EPC levels and the proliferation and migration activity are reduced in patients with CVD, diabetes or hypercholesterolemia.[62-65] Other cardiovascular risk factors such as smoking and CRP are also associated with impaired EPC numbers and function. In the atherosclerotic apoE-/- mouse model systemic transfusion of systemic progenitor cells inhibits the progression of atherosclerotic lesions.[66]

It is apparent that EPC can facilitate endothelial repair and is involved in the development of atherosclerosis. However, it is not clear what the exact contribution of EPC is in cardiovascular diseases.

1.4 Lipid metabolism

Cholesterol and triglycerides are of essential for many different processes in the human body and for energy storage. Since cholesterol and triglycerides are hydrophobic, they are packed into lipoproteins particles for transport in the circulation. Dietary cholesterol and triglycerides are absorbed by the intestines and packed into chylomicrons containing mainly triglyceride (**Figure 3**). Subsequently, these chylomicrons are secreted in the circulations where they acquire apolipoproteins. Once in the circulation, chylomicrons are subjected to lipolysis by endothelium-bound lipoprotein lipase (LPL) resulting in the generation in fatty acid that enters the peripheral tissues for energy storage or source. The chylomicron- remnant particles are further hydrolysed by hepatic lipase (HL) and subsequently taken up by the liver via the low-density lipoprotein receptor (LDLR) or the LDLR-related protein (LRP). The liver plays a central role in the lipid metabolism. The liver processes the cholesterol and triglycerides and secretes these again into the circulation packed into very low-density lipoprotein (VLDL) particles where they acquire apolipoproteins. Similar to chylomicrons, VLDL particles are hydrolysed by LPL and eventually resulting in low-density lipoprotein (LDL) particles. LDL in its turn can be taken up the liver via the LDLR for further processing. LPL is synthesized and secreted by parenchymal cells throughout the body. The activity of LPL is influenced by several apolipoproteins. Apolipoprotein CII serves as a co-factor, whereas apoCI and apoCIII inhibits LPL.[67-70]

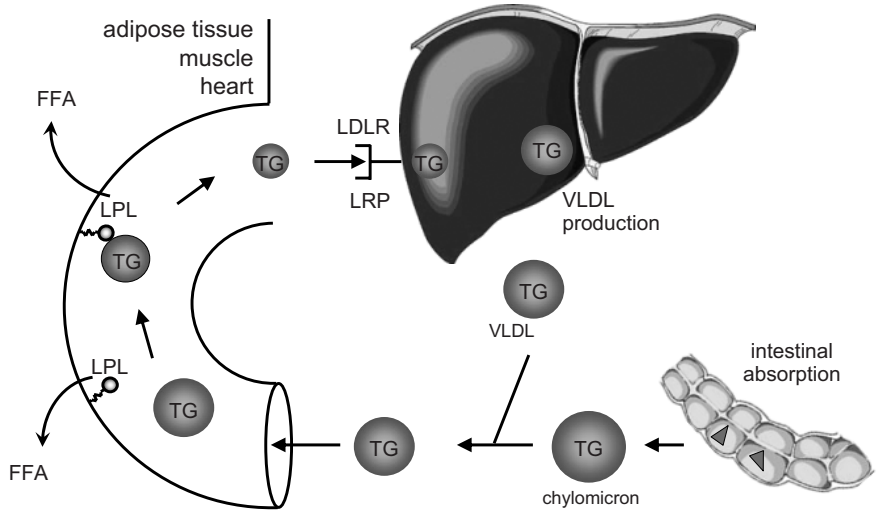


Figure 3 **Lipid metabolism**
See text for explanation. TG: triglyceride, FFA: free fatty acid, LPL: lipoprotein lipase, LDLR: low-density lipoprotein receptor, LRP: low-lipoprotein receptor-related protein, VLDL: very low-density lipoprotein receptor

Originally identified as a member of the LDLR gene family, LRP was suggested to play role in lipid metabolism. *In vitro* studies showed that LRP serves as a receptor for apoE-rich chylomicron remnants and lipoprotein lipases.[71,72]

1.4.1 Low-density lipoprotein receptor-related protein

Structure and expression

The low-density lipoprotein receptor-related protein (LRP) gene is located on chromosome 12 and was identified in 1988 by Herz J *et al*.[73] It is also known as α2-macroglobulin receptor, LRP1 and CD91.[74] LRP consists of 4544 amino acids and is synthesised as a large 600 kDa single polypeptide chain in the endoplasmatic reticulum, which is than cleaved into a 515 kDa and an 85 kDa subunit by furin in the Golgi apparatus. Both subunits remain non-covalently associated where the 515 kDa subunit binds ligands and the 85 kDa subunit is anchored in the plasma membrane. The endoplasmatic reticulum-resident chaperone protein, the 39 kDa receptor- associated protein (RAP) ensures the correct trafficking of LRP along the secretory pathway.[75] Thereby, RAP also promotes proper optimal folding of LRP and prevents premature intracellular binding to its ligands.

LRP is a member of the big low-density lipoprotein (LDL) receptor (LDLR) gene family. This family also includes the LDLR, very low-density lipoprotein (VLDL) receptor (VLDLR), apolipoprotein E receptor 2 (ApoE-R2) and megalin/LRP2/glycoprotein 330 (**Figure 4**). As other members of the LDL receptor gene family LRP contains structural domains that include: a) ligand-binding cysteine-rich complement-type repeats, b) epidermal growth factor (EGF) receptor-like cysteine-rich repeats, c) b-motifs with YWTD repeats, d) transmembrane domain and e) a cytoplasmatic domain that harbours 1-3 NPxY motifs (**Figure 4**).[73] The ligand-binding complement-type repeats are arranged in four different clusters (cluster I, II, III and IV) containing 2, 8, 10 and 11 repeats, respectively. Cluster II and IV bind most of the known ligands. A common feature of most the LDLR gene family members is their ability to bind RAP. RAP antagonizes ligand binding to all members of the LDLR gene family. Therefore, extracellular recombinant RAP is extensively exploited as a tool to study the biology of the LDLR gene family.

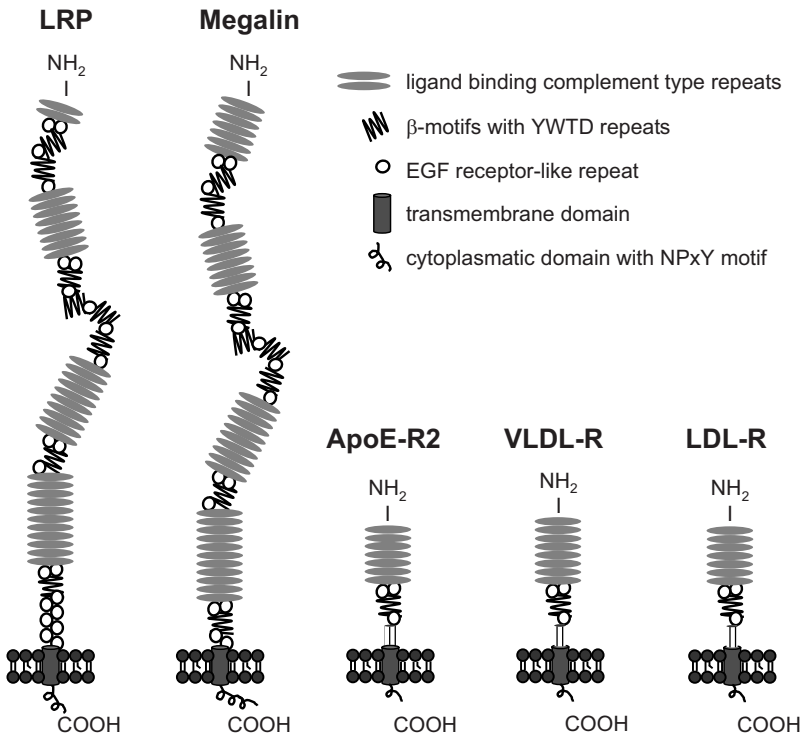


Figure 4 **The low-density lipoprotein receptor gene family**
The LDL receptor gene family consists of several homologous transmembrane receptors involved in endocytosis. All members of the LDL receptor gene family are composed of the same protein domains with similar topological organisations. LDL receptor gene family member include the low-density lipoprotein receptor-related protein (LRP), megalin (pg330 and LRP2), the apolipoprotein E receptor-2 (apoE-R2), the very low-density lipoprotein receptor (VLDLR), and the low-density lipoprotein receptor (LDLR).

LRP is widely expressed in a large variety of tissues. It is abundantly present in the liver, brain, lung, spleen, intestines, reproductive tract and fat tissue.[76] Furthermore, LRP is also expressed in a spectrum of diverse cell types, such as smooth muscle cells, macrophages and fibroblast.

Physiological functions

LRP is a multi-ligand protein. To date, LRP is known to recognise over 50 functionally and structurally numerous ligands (**Table 2**).[77,78] Originally LRP was identified as lipid metabolism receptor. Additionally, LRP is shown to serve as a regulator of the extracellular proteolytic activity by rapid internalising of the uPA/PAI-1 complex in concert with the uPAR and modulating the matrix metalloproteinase levels.[79-82] These evidences imply that LRP is a multifunctional scavenger receptor. Different mice studies confirmed that LRP is indeed

an endocytic scavenger receptor that is not only involved in the lipid metabolism, but also in haemostasis metabolism.[83-85]

Targeted deletion of the LRP gene revealed that LRP is absolutely required in the early embryonic development, suggesting that its physiological role is not restricted as a cargo transporter of extracelluar proteins.[79] The exact mechanism of embryonic lethality is unclear. However, LRP is now known also to be involved in intracellular signalling. It is thought that the cytoplasmatic tail with the NPxY motifs are involved in the interaction with numerous intracellular proteins of the signal transduction pathways.[86] Most of these proteins are adaptor proteins in the regulation of cell signalling, migration and proliferation. Depending on the phosphorylation state of LRP can regulate various intracellular signals in response to different extracellular stimuli by modifying its association with adaptor proteins.[87] LRP is shown to control cell migration and proliferation by phoshorylation in response to PDGF-BB in vascular SMC (VSMC). Failing to control the PDGF signalling in the SMC results in increased atherosclerosis (**see section LRP and atherosclerosis**).[88]

1.4.2 LRP and atherosclerosis

As abovementioned, conventional LRP knockout mice are not viable and die on day 10 of gestation. Therefore, tissue-specific disruption of LRP using the Cre/loxP recombination system has been generated to study the physiological of LRP *in vivo*. Inactivation of hepatic LRP in LDLR deficient mice (MX1Cre LRP$^{flox/flox}$) results in the accumulation of cholesterol-rich remnants lipoproteins suggesting an atherogenic lipid profile.[85] Independent of plasma cholesterol levels these mice show increased atherosclerosis on an atherogenic apoE-/- background.[89]

Next to plasma lipids levels, the proliferation and differentiation of VSCM and macrophages are important in the development of atherosclerosis (see section atherosclerosis). LRP plays a pivotal role in the vascular integrity and the prevention of atherosclerosis in the VSMC.[88] Mice lacking LRP in their VSMC have similar plasma lipid levels as mice with LRP present in the VSMC. However, VSMC LRP deficient mice show increased susceptibility to development atherosclerotic lesions. The elastic layer of the aorta is disrupted. Increase VSMC proliferation and aneurysm formation are observed as a result of abnormal control of the PDGFR expression and activation.

The role of macrophage LRP in the development of atherosclerosis is not fully known. *In vitro* studies implicate that LRP in macrophages has a pro-atherogenic potential. LRP is highly expressed in atherosclerotic lesions and upregulated in macrophages undergoing foam cell formation.[90,91] Additionally, LRP regulates ß2-integrin-mediated adhesion of monocytes to endothelial cells allowing monocytes to migrate into the intima and to differentiate into macrophages.[92] Macrophage LRP has also been demonstrated to play a role in

the translocation of 12/15-lipoxygenase, which stimulates the formation of oxidized LDL.[93,94] In concert with the LDLR, LRP can mediate the uptake of apoE-rich atherogenic lipoproteins into the macrophage.[95-97] Since all these processes promote the formation of foam cells, one would predict that LRP promotes the development of atherosclerosis at the level of macrophages.

Table 2 Extracellular LRP ligands

Lipid metabolism	Growth Factors
Apo E	PDGF
Chylomicron remnants	Midkine
Hepatic lipase	Connective tissue growth factor
Lipoprotein lipase	TGF-β
Lipoprotein (a)	
β-VLDL	*Infection and immunity*
Saposin	Aminoglycosides
Sphingolipid activator protein	Circumsporozoite protein
	Complement C3
Protease and protease/inhibitor complexes	Gentamicin
Activated α2-M*	HIV-Tat protein
Aprotinin	Lactoferin
C1s/C1q inhibitor	Minor group rhinovirus
Elastsae/α1-anti-trypsin	Polymyxcin B
FIXa	Pseudomonas exotoxin A
FVIIa/TFPI	Ricin A
FVIIIa	Saposin
FXa/TFPI	Trichosanthin
FXIa/protease-1	
Neuroserpin	*Matrix proteins*
Neuroserpin/tPA	Fibronectin
PAI-1	MMP-13
PAI-1/thrombin	MMP-9
PAI-1/tPA	TSP-1
PAI-1/uPA	TSP-2
Pregnancy zone protein/protease complexes	TSP-2/MMP-2
Pro-uPA	
TFPI	*Others*
Thrombin/anti-thrombin III	Amyloid precursor protein
Thrombin/heparin cofactor II	Amyloid β-chain
Thrombin/proteinase nexin-1	Calreticulin
tPA	Collectins
Trypsin/α1-anti-trypsin	HSP-96
TSP-2/MMP-2	RAP
uPA	
uPA/protease nexin-1	
α2-M*/protease complexes	

1.5 Outline of this thesis

In this thesis we aimed to expand our knowledge on the pathophysiological aspects of the metabolic syndrome in transgenic mice. The metabolic syndrome involves multiple aspects and has a major impact on cardiovascular diseases. In the first part of thesis the role of PAI-1 in the development of insulin resistance will addressed. This part will also focus on the mechanism of plasma PAI-1 clearance. Plasma PAI-1 is increased in patients with the metabolic syndrome. Obesity and insulin resistance are key components of the metabolic syndrome. The increased plasma PAI-1 levels are suggested to be the result of increased expression in the vascular endothelium, adipose tissue and liver. However, it is not known if the clearance also contributes to the increased plasma PAI-1 levels. **Chapter 2** describes the clearance and plasma levels of PAI-1 in a genetically and a diet-induced insulin resistant mouse models. A number of studies have shown that LRP can bind, internalise and degrade PAI-1 *in vitro*. However, it is not known whether LRP indeed plays a role in the clearance of plasma PAI-1 *in vivo*. **Chapter 3** addressed the role of hepatic LRP in the regulation of plasma PAI-1 *in vivo*. For this purpose, we studied the clearance of PAI-1 in hepatic LRP deficient mice under different conditions.

In the second part of this thesis, the roles of LRP in atherosclerosis and LPL activity in lipid metabolism are addressed. Hepatic LRP deficient mice have elevated fasted plasma cholesterol and triglyceride levels, mainly present as VLDL particles on a LDLR-/-VLDL-/- background. Since VLDL is continuously produced in the liver, VLDL remnants still need to be cleared to maintain a steady state level. **Chapter 4** addressed the whether LPL activity is important for the hepatic clearance of VLDL remnants independent of the three major apoE-recognizing receptors LRP, LDLR and VLDLR. LRP in the liver and SMC is shown to have atheroprotective role. Macrophages play a key role in the development of atherosclerosis next to SMC. Data from several *in vitro* studies suggest a pro-atherogenic of LRP in the macrophage. In **chapter 5** we investigated the role macrophage LRP in the development of atherosclerosis *in vivo*.

Finally, role of low-grade inflammation in endothelial restoration is addressed. Subjects with the metabolic syndrome have chronic low-grade inflammation and increased risk for cardiovascular diseases. **Chapter 6** describes the influence of low-grade inflammation on the number of EPC in patients with the metabolic syndrome. The association between the number of EPC and the extent of atherosclerosis in the carotid artery is also described.

The results obtained from these studies and the implications for future research are discussed in **chapter 7**.

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Chapter 2

The clearance of plasma PAI-1 is not affected in insulin resistant mice

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Submitted for publication

Summary

Increased plasma PAI-1 levels are observed in insulin resistance human subjects. It is thought that increased plasma PAI-1 levels can predict the incident of insulin resistance. This is supported by several rodent mouse models. Mice lacking PAI-1 do not develop insulin resistance. However, others showed that PAI-1-deficient mice have more adipose tissue and a worsened metabolic profile. Therefore, it is not fully understood how PAI-1 is involved in insulin resistance. In the present study we investigated 1) the plasma PAI-1 levels in diet-induced insulin resistant mice in time and 2) the contribution of the clearance of PAI-1 to the increased plasma PAI-1 levels in insulin resistant mice. We found that plasma PAI-1 levels increase in diet-induced insulin resistance and that these increased plasma PAI-1 levels follow rather than precede insulin resistance. Insulin resistance was already present after 4 weeks of high fat diet, whereas increased plasma PAI-1 levels were observed only after 12 weeks of high fat diet. Furthermore, we showed that the clearance of PAI-1 does not contribute to the increased plasma PAI-1 levels in both diet-induced and genetically insulin resistance mice. Taken together, our data support the concept that PAI-1 is not causally involved in the development of insulin resistance.

Introduction

Plasminogen activator inhibitor-1 (PAI-1) is the main physiological inhibitor of tissue-type plasminogen activator (t-PA). Increased plasma PAI-1 levels are associated with decreased fibrinolysis.[1] Several epidemiological studies have shown strong association between increased plasma PAI-1 levels on one hand, and obesity and insulin resistance on the other.[2-5] Progression of PAI-1 plasma levels in addition to initial high plasma levels are associated with incident of diabetes.[6] However, the exact mechanism of increased PAI-1 insulin resistance are incompletely understood.

The expression of PAI-1 in adipose tissues is positively correlated with obesity in human and rodents,[7-11] suggesting a possible role in the development of obesity and insulin resistance. However, animal studies on the role of PAI-1 in insulin resistance show contradicting data. Disruption of the *pai-1 gene* in ob/ob mice reduces adiposity and improves the metabolic profile as determined by glucose and insulin tolerance test.[12] Additionally, PAI-1-deficient mice do not develop diet-induced obesity and insulin resistance. Administration of synthetic PAI-1 inhibitor induces higher insulin sensitivity in WT mice.[13,14] These studies suggest that PAI-1 may not merely increase in response to obesity and insulin resistance, but may have direct causal role in obesity and insulin resistance. In contrast to these studies, others showed that PAI-1-deficient mice develop more adipose tissue.[15] In agreement with this, transgenic mice overexpressing PAI-1 have a lower body weight, lower adipose tissue mass, intraperitoneal fat and an improved metabolic profile.[16] Taken together, it is still not apparent how PAI-1 is involved in obesity and insulin resistance.

PAI-1 is known to be synthesized by various tissues including liver and adipose tissue. Increased plasma PAI-1 levels can result from increased expression from the adipose tissue. Increased mRNA expression of PAI-1 is positively correlated with obesity in human and rodents.[7,8,11] Alternatively, increased plasma PAI-1 can result from decreased plasma PAI-1 clearance. We previously showed that increased plasma PAI-1 levels result from decreased clearance in mice overexpressing receptor-associated protein, the low-density lipoprotein receptor gene family.

In the current study, we investigated the plasma PAI-1 levels during the development of insulin resistance. Additionally, we studied the contribution of the clearance of PAI-1 in insulin resistance. For this purpose, we used both diet-induced and genetically insulin resistant mouse models. Here, we show that the plasma PAI-1 levels increase in insulin resistance. Moreover, the increased plasma PAI-1 levels follow rather than precede insulin resistance. Furthermore, we also showed that the increased plasma PAI-1 levels are not due to delayed clearance in both diet-induced and genetically obese insulin resistant mice. Our data do not support the concept that PAI-1 has a direct causal role in insulin resistance. Plasma PAI-1 levels merely increase in response to insulin resistance

Material and Methods

Animals and diet

Twelve weeks old male wild-type C57Bl/6 mice (Charles River, Maastricht, The Netherlands) were housed in a temperature and humidity-controlled room on a 12:12-h light-dark cycle. Mice were fed a high fat diet (45 energy%, HFD) or a control diet (10 energy%, control) with fat derived from palm oil (Hope Farms, Woerden, The Netherlands). Male db/db mice (Charles River, Maastricht, The Netherlands) and their respective C57Bl/6 control mice were fed regular chow diet. Mice had free access to water.

All animal experiments were approved by the Animal Ethics Committee from the Leiden University Medical Center, Leiden, The Netherlands.

Blood sampling and analysis

For glucose and insulin measurements, blood was collected in EDTA-coated vials by tail bleeding. For PAI-1 antigen measurements, blood samples were obtained collected in vials containing 1/10 volume of 3.2% (w/v) citrate. Plasma was prepared by centrifugation (8000x*g* for 10 minutes at 4°C), snap-frozen and stored at -80°C prior to analysis. Mouse plasma PAI-1 antigen (Innovative Research, CA) was determined by enzyme-linked immunosorbent assay (ELISA) according to manufacturer's instructions. Plasma glucose was determined using commercially available kits (Instruchemie, Delftzijl, The Netherlands). Insulin was determined by a mouse insulin ELISA (Mercodia, Uppsula, Sweden). Exogenous PAI-1 decay experiments were performed as previously described.[17] In short, mice received a bolus of 1 μg/mouse purified latent murine PAI-1 (Innovative research, CA) via the tail vein. Values are expressed as percentage of PAI-1 remaining in the circulation, with the amount of PAI-1 present at 1 minute after injection considered as 100%. An one phase exponential fit was used to calculate the half-lifes (t1/2).

Hyperinsulinemic euglycemic clamp experiments

Hyperinsulinemic euglycemic clamps experiments were performed as described.[18] Mice were fasted overnight with food withdrawn at 5 p.m. the day prior to the experiments. Mice were anesthetised with 6.25 mg/kg acepromazine (Alfasan, Woerden, The Netherlands), 6.25 mg/kg midazolam (Roche, Mijdrecht, The Netherlands) and 0.31 mg/kg fentanyl (Janssen-Cilag, Tilburg, The Netherlands). Basal glucose turnover was determined by a continuous infusion of ^{14}C-glucose (GE Healthcare, Little Chalfont, U.K.) for 60 minutes. Subsequently, insulin was administered for 90 minutes to attain steady state circulating insulin levels of ~4 ng/ml. A 12.5% D-glucose solution was used to maintain euglycemia as determined at 10 min intervals via tail bleeding with a hand glucose monitor (Accu-chek, Sensor Comfort, Roche Diagnostics GmbH, Mannheim, Germany). Blood samples (60 μl) were taken during

the basal period (after 50 and 60 min) and during the hyperinsulinemic period (after 70, 80, and 90 min) to determine plasma concentrations of glucose and insulin.

Statistical analysis

Data are analysed by means of the Mann-Whitney *U* test. $P < 0.05$ was regarded as statistically significant.

Results

Body weight, plasma glucose and insulin levels in mice on a high fat diet

Male C57B1/6 mice were fed a high fat diet (HF) to induce insulin resistance. The body weight was determined at baseline, and at 4 and 12 weeks after high fat diet (HFD) or control diet. A significant increase in body weight in the HFD group was already observed from 4 weeks of HFD on (Figure 1). To confirm insulin resistance, we performed hyperinsulinemic euglycemic clamp analyses.[18] The glucose infusion rate (GIR) was significantly lower in mice fed the HFD after 4 and 12 weeks as compared to mice fed the control diet, confirming rapid onset (*i.e.* 4 weeks) of insulin resistance (Figure 2).

Plasma PAI-1 levels and clearance in diet-induced insulin resistant mice

Plasma PAI-1 levels were measured in time to study when plasma PAI-1 levels will increase during the development of insulin resistance. Plasma PAI-1 levels were not affected until 12 weeks of HFD feeding. In HFD-induced insulin resistant mice, plasma PAI-1 levels were similar between the control and HFD groups after 4 and 8 weeks of diet (Figure 3). However, after 12 weeks of HFD plasma PAI-1 levels were significantly increased as compared to the control diet (Figure 3). This increase was still present after 16 weeks of HFD. We next examined whether altered clearance of PAI-1 contributed to the observed increased plasma PAI-1 levels under prolonged HFD feeding conditions. Plasma PAI-1 clearance of intravenously administered purified murine PAI-1 were studied in diet-induced insulin resistant mice after 4 and 16 weeks of HFD or control diet. Not surprisingly, the plasma PAI-1 decay after 4 weeks of diet was not different between the control and HF diet groups, since plasma PAI-1 levels were similar between the groups (Figure 4). The half-lives at 4 weeks of diet were 12.9 ± 4.7 and 9.8 ± 3.0 minutes for control and HF group, respectively. However, after 16 weeks of HFD, the decay was also not affected by HFD as compared to control diet (Figure 4). The half-lives were 8.5 ± 1.6 and 7.9 ± 1.4 for HFD and control diet, respectively. Taken together, the increase of plasma PAI-1 levels followed the development of insulin resistance and this increase was not the consequence of decreased plasma clearance.

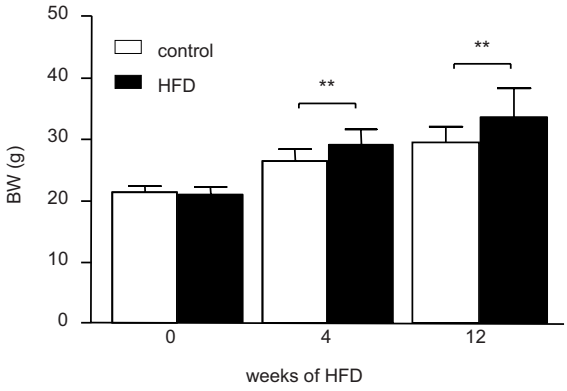

Figure 1 **Body weight**
Body weight of twelve weeks old male C57Bl/6 mice (Charles River, Maastricht, The Netherlands) at base line, 4 and 12 weeks of HFD (45 energy%) or control diet (10 energy%). The HFD and the control diet group are depicted by black and white bars, respectively. **P < 0.01, significantly different from the control group.

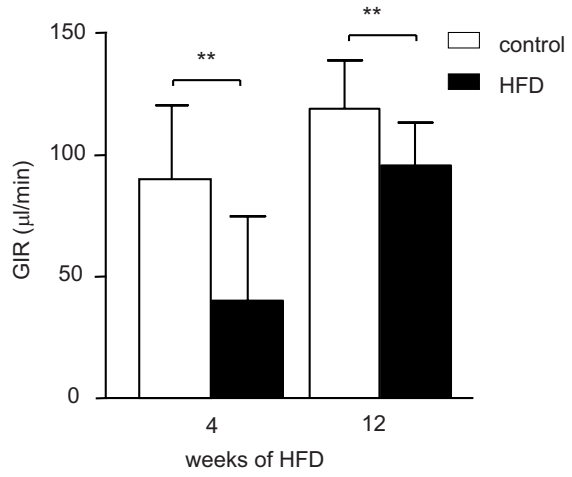

Figure 2 **Glucose infusion rate**
Glucose infusion rate as measured by hyperinsulinemic euglycemic clamp after 4 and 12 weeks of diet. GIR: glucose infusion rate. . **P < 0.01, significantly different from the control group.

Plasma PAI-1 levels and clearance in genetically insulin resistant mice

To investigate whether elevated plasma PAI-1 levels were related to genetically insulin resistance, we measured plasma PAI-1 levels and plasma PAI-1 clearance in db/db mice. The body weight of db/db mice was significantly higher as compared to wild-type C57B6/J mice (48.5 ± 3.0 vs. 22.9 ± 1.3, $P < 0.0001$). Plasma PAI-1 levels were about 5-fold higher in the genetically insulin resistant db/db mice as compared to wild-type C57B6/J mice consistent with previous findings (Figure 5A).[19]

Similar as in the diet-induced obese insulin resistance mice, plasma PAI-1 clearance was similar between the db/db and wild-type C57B6/J mice (Figure 5B). The plasma PAI-1 half-lives were 10.7 ± 5.5 and 9.1 ± 2.4 minutes for db/db and wild-type C57B6/J mice, respectively ($P = 0.56$).

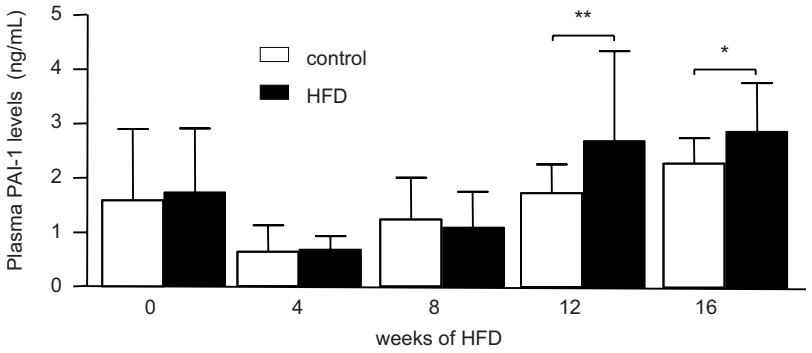

Figure 3 **Plasma PAI-1 levels in time**
Plasma PAI-1 levels at t = 0, 4, 8, 12 and weeks of diet. *P < 0.05, **P < 0.01, significantly different from the control group.

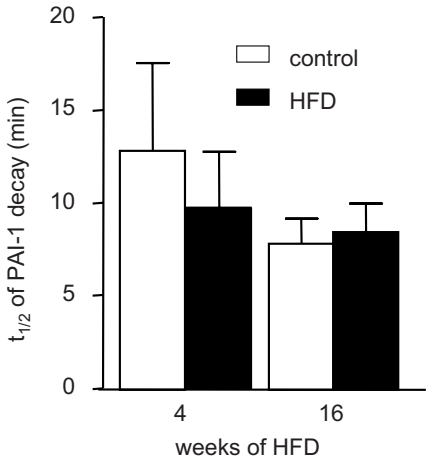

Figure 4 **Plasma PAI-1 clearance in diet-induced insulin resistant mice**
Plasma PAI-1 half-lives of diet-induced insulin resistant mice. A one-exponential fit was used to calculate the half-lives, considering the amount of PAI-1 present at 1 minute after injection as 100%.

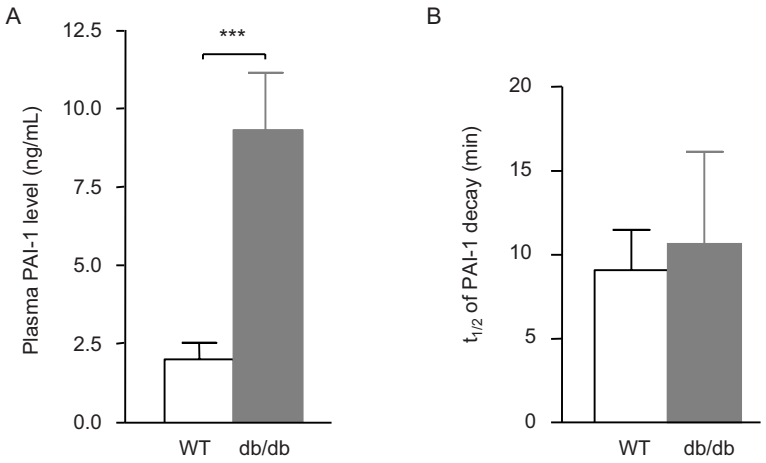

Figure 5 **Plasma PAI-1 levels and clearance in genetically insulin resistant db/db mice**
(A) Plasma PAI-1 levels of genetically insulin resistant 12-weeks old db/db mice (grey bars) and control wild-type (WT) C57Bl/6 mice (white bars). (B) Plasma PAI-1 half-lives of db/db and WT mice. ***P < 0.001, significantly different from control WT C57Bl/6J mice.

Discussion

The aim of the current study was to investigate the plasma PAI-1 levels in the development of insulin resistant mice. In addition, the clearance of plasma PAI-1 levels was studied in diet-induced and genetically insulin resistant mice. We showed that the increase of plasma PAI-1 levels follows the insulin resistance rather than preceding insulin resistance. Furthermore, the increased plasma PAI-1 levels do not find its origin in delayed clearance.

Several epidemiological studies have shown that plasma PAI-1 can predict the development of diabetes independently from other known risk factors.[3,4] The progression of PAI-1 plasma levels in addition to initial high plasma levels is thought to be associated with incident diabetes.[6] PAI-1-deficient mice do not develop insulin resistance and have improved metabolic profiles, suggesting a causal relation between PAI-1 and insulin resistance.[13,14] However, our data do not support these previous findings. We showed that the increase of plasma PAI-1 levels follows insulin resistance rather than precede insulin resistance. Insulin resistance was already present as early as 4 weeks of HFD, whereas plasma PAI-1 levels increased only after 12 weeks of HFD. Lower insulin levels and higher glucose levels were observed in mice overexpressing PAI-1.[16] The PAI-1 deficient mouse model by Morange *et al.* [15] has improved metabolic profiles. These data together with our results suggest that PAI-1 does not have a causal role in the development of insulin resistance. Increased plasma PAI-1 levels may be of clinical relevance, but a pathophysiologically epiphenomenon

of the inflammatory setting of insulin resistance. Improvement of the insulin resistance and thereby the inflammatory setting may also result in decreased plasma PAI-1 levels.[24]

The increased plasma PAI-1 in insulin resistance is thought to be the result of increased expression by the adipose tissue.[20,21] However, decreased plasma PAI-1 clearance may also result in increased plasma PAI-1 levels. We now show that the clearance of PAI-1 in both genetically and diet-induced insulin resistant mice models, does not contribute to the plasma PAI-1 levels. Therefore, the increased plasma PAI-1 levels are most likely the result of increased expression. PAI-1 is expressed in several tissues, including the adipose tissue, the liver and the endothelium. The expression of PAI-1 in the liver was similar between insulin resistant and control mice (data not shown). Insulin resistance is associated with endothelial dysfunction.[22,23] The main physiological function of PAI-1 is the inhibition of fibrinolysis of a thrombus present in the blood vessel after endothelial damage. Therefore, the vascular endothelium might also be an important source of increased PAI-1 expression in insulin resistance in addition to the adipose tissue.

In conclusion, our data demonstrate that increased plasma PAI-1 levels follow insulin resistance rather than precede insulin resistance. This plasma PAI-1 elevation does not find its origin in delayed plasma clearance.

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Chapter 3

Plasma PAI-1 level is not regulated by the hepatic low-density lipoprotein receptor-related protein

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J. Throm Haemost 2007, 11:2301-4

Abstract

Increased plasma levels of plasminogen activator inhibitor-1 (PAI-1) are associated with increased obesity, insulin resistance and cardiovascular diseases. While research has been directed towards the production of PAI-1, the clearance of PAI-1 remains poorly understood. *In vitro* studies have demonstrated that PAI-1 is bound, internalised and degraded by the low-density lipoprotein receptor (LDLR)-related protein (LRP). In the present study, we have investigated the role of hepatic LRP in the clearance of plasma PAI-1 *in vivo,* employing mice conditionally lacking hepatic LRP (LRP-). Plasma PAI-1 levels were similar between LRP- and control LRP+ littermates. LRP status also did not affect the clearance of both exogenously infused purified murine PAI-1 and endogenously endotoxin-stimulated PAI-1. Remarkably, adenovirus-mediated gene transduction of the LDLR gene family antagonist receptor-associated protein (RAP) resulted in a significant increase of plasma PAI-1 in both LRP+ and LRP- mice. In addition, the plasma PAI-1 decay was prolonged 2-fold in mice overexpressing RAP in the circulation. The plasma levels of PAI-1 in LDLR-/-, VLDR-/-, double deficient LRP-LDLR-/- and LRP-VLDLR-/- were not different from plasma PAI-1 levels in LRP+ mice. Therefore, we conclude that in contrast to the *in vitro* data, hepatic LRP does not contribute to the clearance of plasma PAI-1 to a significant extent. In addition, we propose that RAP-sensitive mechanisms other than hepatic LRP, LDLR and VLDLR are involved in the clearance of PAI-1 *in vivo*.

Keywords: PAI-1, LRP, clearance, mice

Introduction

Plasminogen activator inhibitor-1 (PAI-1) is the main physiological inhibitor of tissue-type and urokinase-type plasminogen activator (tPA, uPA). Increased plasma PAI-1 levels are strongly associated with obesity, diabetes and cardiovascular diseases.[1,2] Furthermore, increased plasma PAI-1 levels are associated with decreased fibrinolysis.[3] This increase is associated with enhanced PAI-1 expression in vascular endothelium, adipose tissue and liver.[1] Alternatively, decreased plasma PAI-1 clearance might contribute to the increased plasma PAI-1 levels. However, it remains unknown how PAI-1 is cleared from the circulation and to what extent decreased plasma PAI-1 removal contributes to increased plasma PAI-1 levels.

PAI-1 interacts with the low-density lipoprotein receptor (LDLR)-related protein (LRP) in vitro.[4] LRP is a multi-ligand endocytic receptor of the LDLR gene family, which also includes LDLR and very low-density lipoprotein receptor (VLDR). All ligand binding to LDLR gene family members is antagonised by the receptor-associated protein (RAP). LRP is a multi-ligand multifunctional receptor. It recognizes >30 structurally and functionally different ligands in vitro, including PAI-1.[4,5] PAI-1 contains binding sites for the low-density lipoprotein receptor (LDLR)-related protein (LRP).[6] *In vitro* studies have demonstrated that PAI-1 is bound, internalised and degraded by LRP.[7] Multiple in vitro studies have shown that PAI-1 in complex with its target proteins is a better ligand for LRP than PAI-1 alone. However, PAI-1 binds to LRP with similar affinity as factor VII (FVII), which is demonstrated to be regulated by LRP *in vivo*.[6,8]

In the present study, we studied the role of hepatic LRP in the regulation of plasma PAI-1 levels in vivo. To this end, we used the unique mouse model that allows Cre/loxP-mediated deletion of hepatic LRP.[9] In addition, we have addressed whether other RAP-sensitive mechanisms are involved in the clearance of plasma PAI-1 levels using adenovirus-mediated gene transfer of RAP. We propose that RAP-sensitive pathways other than hepatic LRP, LDLR and VLDLR are involved in the clearance of plasma PAI-1 in mice.

Material and Methods

Plasma PAI-1 clearance in transgenic mice

We employed LRP, LDLR and VLDR deficient mice and combination thereof.[9-11] Age-matched 8-12-weeks old mice homozygous for the "floxed" LRP allele, either with or without the MX1Cre transgene ($MX1Cre^{+}LRP^{flox/flox}$ or $LRP^{flox/flox}$, respectively) littermates were used. LRP deficiency was induced as described.[8,9] In clearance experiments, male mice received a bolus of 1 μg/mouse purified latent murine PAI-1 (Innovative Research, CA) via the tail vein. Values are expressed as percentage of PAI-1 remaining in the circulation, with the amount

of PAI-1 present at 1 minute after injection considered as 100%. Data were corrected for endogenous PAI-1 levels. A one phase exponential fit was used to calculate the half-lives. For endogenous PAI-1 turnover, female mice received 5 μg of endotoxin (LPS Re 595, Sigma, MO) intraperitoneally as described.[12] All animal experiments were approved by the institutional committees on animal welfare of TNO-Quality of Life.

Plasma analysis

Blood samples were obtained by tail bleeding and collected in tubes containing 1/10 volume of 3.2% (w/v) citrate. Plasma was prepared by centrifugation (8000x*g* for 10 minutes at 4°C), snap-frozen and stored at -80°C prior to analysis. Mouse plasma PAI-1 antigen (Innovative Research, CA) and serum amyloid A (SAA; Biosource Europe, Belgium) were measured by enzyme-linked immunosorbent assay according to manufacturers instructions. Mouse plasma FVIII activity was measured using an one-stage coagulation assay as described.[13] Pooled plasma of C57BL/6J mice was used as reference.

Recombinant adenovirus transduction

Recombinant adenovirus ($1x10^9$ plaque-forming units) containing RAP (Ad-RAP) or β-galactosidase cDNA (Ad-β-Gal) were used for *in vivo* transduction as described.[14] Ad-RAP gene transduction results in hepatic overexpression of secretable RAP in plasma. Blood samples were collected 8 days after adenovirus injection. The PAI-1 decay experiments were performed at 8 days after virus injection. Mice intravenously received a bolus of purified murine PAI-1 (1 μg per mouse) and plasma elimination of PAI-1 was followed in time. The functionality of Ad-RAP was evaluated by measuring plasma cholesterol levels in LDLR-/- mice as described.[14]

Statistical analysis

Data are represented as geometric means and 68% confidence intervals (CI), which represent one standard deviation from the geometric mean if a log-normal distribution is assumed. Data are analyzed by means of the Mann-Whitney *U* test. $P < 0.05$ was regarded as statistically significant.

Results and Discussion

Plasma PAI-1 levels and clearance in LRP deficient mice

In vitro studies have shown that LRP plays a major role in the catabolism PAI-1. To explore the physiological relevance of hepatic LRP in the regulation of plasma PAI-1 clearance, we measured plasma PAI-1 in induced $MX1Cre^{+}LRP^{flox/flox}$ (LRP-, n = 31) mice and control $LRP^{flox/flox}$ (LRP+, n = 33) littermates (Figure 1A). LRP- mice displayed similar plasma levels

as controls. Plasma PAI-1 levels were 1.3 (1.2-1.5) ng/mL and 1.6 (1.4-1.8) ng/mL for LRP- and control LRP+ littermates, respectively (p = 0.19). Significant increase in plasma FVIII activity was observed in these LRP- [5.4 (5.0-5.9) U/mL] and LRP+ [2.7 (2.1-3.4) U/mL] mice (P < 0.05), which is consistent with our previous findings, indicating adequate induction of LRP deficiency (Figure 1B).[8] To further investigate whether LRP contributes to the clearance

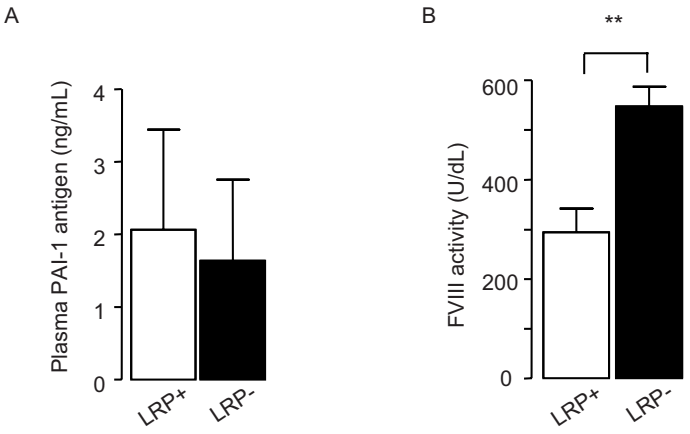

Figure 1 **Plasma PAI-1 antigen and FVII activity in hepatic LRP deficient mice LRP.**
Plasma PAI-1 antigen levels (A) and FVII activity (B) in LRP deficient (LRP-, n = 31) and control littermates (LRP+, n = 33). **p < 0.01, significantly different from control LRP+ littermates.

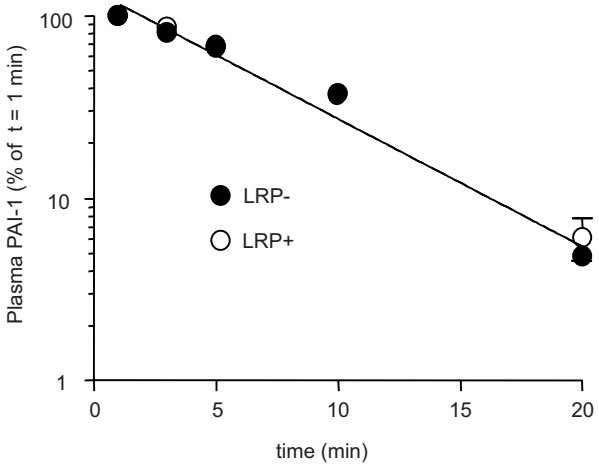

Figure 2 **Plasma PAI-1 clearance in hepatic LRP deficient mice**
LRP- and control LRP+ littermates (n = 6) intravenously received a bolus of purified mice PAI-1 (1 μg/mouse) and the plasma elimination of PAI-1 was followed in time. A one-exponential fit was used to calculate the half-lives, considering the amount of PAI-1 present at 1 minute after injection as 100%.

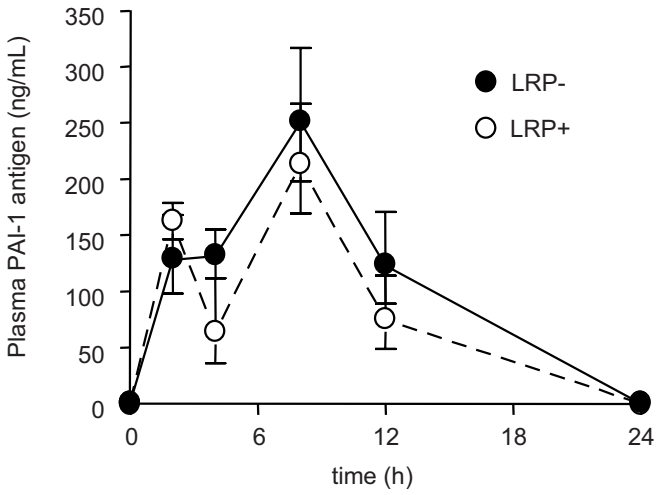

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Figure 3 **Plasma PAI-1 antigen and clearance of plasma PAI-1 upon LPS challenge**
LRP- (n = 8) and control LRP+ (n = 7) littermates intraperitoneally received 5 μg/mouse endotoxin LPS. Subsequently, plasma PAI-1 was measured during 24-hours.

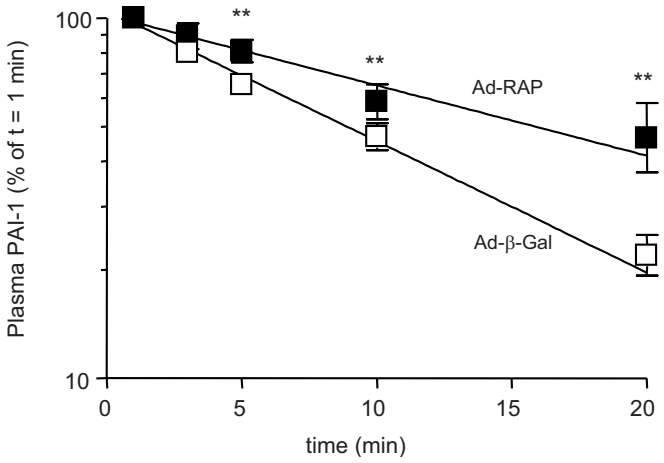

Figure 4 **Plasma PAI-1 clearance in mice overexpressing RAP**
Mice intravenously received 1 x 109 plaque forming units of recombinant Ad-β-Gal or Ad-RAP (n = 6). Eight days after adenovirus administration, mice intravenously received a bolus of purified murine PAI-1 (1 μg/mouse) and the plasma elimination of PAI-1 was followed in time as in figure 2. **p < 0.01, significantly different from Ad-β-Gal treated mice.

of PAI-1, we studied the plasma elimination of intravenously administered purified murine PAI-1. PAI-1 half-lives were identical between LRP- and LRP+ littermates (Figure 2). The half-lives were calculated to be 4.3 (3.7-4.3) minutes and 4.3 (3.7-4.3) minutes for LRP- and LRP+, respectively.

It has been established that lipopolysaccharide (LPS) can induce increased plasma PAI-1 levels in mice.[12] Therefore, we challenged LRP- and LRP+ mice with endotoxin to induce a transient rise in endogenous plasma PAI-1 levels. Indeed, upon LPS challenge a transient rise in endogenous plasma PAI-1 levels was observed (Figure 3). More importantly, the subsequent plasma PAI-1 elimination in LRP- and LRP+ littermates was similar (Figure 3). The areas under the curve were 2.8 (2.3-3.5) μg/mL.h and 2.1 (1.6-2.8) μg/mL.h for LRP- and LRP+ mice, respectively (p = 0.61). These data indicated that hepatic LRP is not involved in the regulation of plasma PAI-1 levels to a significant extent *in vivo*.

Adenovirus-mediated overexpression of RAP in hepatic LRP deficient mice

In vitro studies have previously demonstrated that the LDLR gene family antagonist receptor-associated protein (RAP) inhibits the endocytosis and degradation of PAI-1.[15] Hence, we investigated whether RAP-dependent mechanisms other that hepatic LRP are involved in the regulation of plasma PAI-1 levels. Administration of adenovirus containing RAP cDNA (Ad-RAP) evoked a significant increase in plasma PAI-1 levels in both LRP+ and LRP- mice, as compared to mice that received control adenovirus containing β-galactosidase cDNA (Ad-β-Gal, Table 1). However, no difference in plasma PAI-1 levels was observed between LRP+ and LRP- mice following Ad-RAP administration (P = 0.49). As plasma PAI-1 levels also increased after Ad-β-Gal administration, we considered the possibility that the plasma PAI-1 increase is due to an acute phase reaction upon adenovirus administration. Therefore, we measured the acute phase protein SAA. Indeed, adenoviral administration resulted in a significantly increased SAA. However, the increased SAA was more pronounced in mice that received Ad-β-Gal as compared to Ad-RAP. Plasma SAA levels were 2.1 (0.8-5.1) μg/mL and 99.7 (82.3-120.8) μg/mL for Ad-RAP and Ad-β-Gal, respectively (P < 0.05). This strongly suggests that the increased plasma PAI-1 in mice overexpressing RAP is independent of the systemic inflammatory response to adenovirus. Of note, although injection were standardized according to the plaque forming units, the absence of a SAA elevation following Ad-RAP likely reflects difference in Ad-β-Gal and Ad-RAP batches with regard to the content of non-infectious viral particles. However, we cannot fully exclude the possibility that RAP itself modulates SAA levels.

Table 1 **Plasma PAI-1 in control, LRP-, LDLR-/-, VLDLR-/-, LRP-LDLR-/- and LRP-VLDLR-/- mice with and without adenovirus-mediated overexpression of RAP.**

genotype	adenovirus	PAI-1 (ng/mL)	n
LRP+	-	1.6 (1.4-1.8)	33
LRP-	-	1.3 (1.2-1.5)	31
LRP+	ad-β-Gal	32.5 (24.9-42.3)	4
LRP+	ad-RAP	159.3 (98.9-256.6)*	4
LRP-	ad-β-Gal	19.6 (11.7-33.0)	4
LRP-	ad-RAP	261.9 (192.2-357.0)#	4
LDLR-/-	-	1.5 (1.4-1.6)	14
VLDLR-/-	-	2.1 (1.8-2.3)	5
LRP-LDLR-/-	-	1.6 (1.4-1.8)	16
LRP-VLDLR-/-	-	1.7 (1.5-2.0)	7

For the adenovirus-mediated overexpression of RAP, mice received 1x109 plaque forming units recombinant adenovirus containing RAP (Ad-RAP) or control β-galactosidase (Ad-β-Gal) cDNA. Blood samples were collected 5 days after adenovirus administration. Blood samples were then analysed for PAI-1. Data represent geometric mean with 68% CI. *$p < 0.05$, significantly different from ad-β-Gal injected LRP+ mice. #$p < 0.05$, significantly different from ad-β-Gal injected LRP- mice.

To study whether the increased plasma PAI-1 levels in Ad-RAP treated mice can be attributed to impaired clearance, we followed the clearance of exogenously injected PAI-1 in these mice. The clearance of PAI-1 was 2-fold slower in mice overexpressing RAP (Figure 4). The half-lives were calculated to be 15.4 (13.3-18.4) minutes in Ad-RAP treated mice and 8.3 (7.6-9.1) minutes in mice that received Ad-β-Gal ($P < 0.01$). These data indicate that RAP-dependent mechanisms other than hepatic LRP are involved in the regulation of PAI-1 *in vivo*.

RAP-sensitive receptors include LDLR and VLDLR. Therefore, we measured plasma PAI-1 levels in LDLR-/-, VLDLR-/-, and double deficient LRP–LDLR-/- and LRP– VLDLR-/- mice. Plasma PAI-1 levels in these mice were not different from plasma PAI-1 in LRP+ mice (Table 1), suggesting that neither LDLR nor VLDLR is critically involved in the regulation of plasma PAI-1 levels. Additional studies are required to establish which RAP-sensitive mechanisms are involved in the regulation of plasma PAI-1 levels. The question remains whether the LRP/PAI-1 interaction is of any physiological importance. It could be possible that the interaction between LRP and PAI-1 is of importance only when PAI-1 is in complex with its target proteases. The high affinity LRP binding site in PAI-1 is demonstrated to be exposed when PAI-1 is in complex with t-PA.[4] However, the similar plasma PAI-1 levels between LRP- and LRP+ (Figure 1) are a strong argument against a significant accumulation of plasma PAI-1 complexes in the present study. Alternatively, the PAI-1/LRP interaction might only be of importance in cellular signaling locally. It has been shown that PAI-1 is a potent chemoattractant molecule, an activity that depends on the interaction with LRP for cell signalling.[16] Identification of the molecular mechanisms that underlie the regulation of PAI-1 levels in the circulation may further advance our understanding of increased plasma PAI-1 levels in patients with obesity, diabetes and cardiovascular diseases.

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Acknowledgement

We would like to thank N. van Tilburg for his excellent technical support.

Chapter 4

The hepatic uptake of VLDL in lrp-ldlr$^{-/-}$vldlr$^{-/-}$ mice is regulated by LPL activity and involves proteoglycans and SR-BI

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J. Lipid Res (2008) 49:1553-61

Abstract

Lipoprotein lipase (LPL) activity plays an important role in preceding the remnant clearance via the three major apoE-recognizing receptors, the LDL receptor (LDLr), LDLr related protein (LRP), and VLDL receptor (VLDLr). We recently showed that *lrp*$^{-}$*ldlr*$^{-/-}$*vldlr*$^{-/-}$ mice have elevated fasted plasma total cholesterol (TC) and triglyceride (TG), mainly present as VLDL. However, since VLDL is continuously produced by the liver, their core remnants must thus still be cleared to attain steady state lipid levels in plasma. The aim of this study was to determine whether LPL activity is important for the clearance of VLDL core remnants irrespective of these receptors, and to determine the mechanisms involved in the hepatic uptake of these remnants. Administration of an adenovirus expressing LPL (Ad*LPL*) into *lrp*$^{-}$*ldlr*$^{-/-}$*vldlr*$^{-/-}$ mice reduced both VLDL-TG and VLDL-TC levels. Conversely, inhibition of LPL by Ad*APOC1* increases plasma VLDL-TG and VLDL-TC levels. Metabolic studies with radiolabeled VLDL-like emulsion particles showed that the clearance and hepatic association of their core remnants positively correlated with LPL activity. This hepatic association was independent of the bridging function of LPL and HL, since heparin did not reduce the liver association. *In vitro* studies demonstrated that VLDL-like emulsion particles avidly bound to the cell surface of primary hepatocytes from *lrp*$^{-}$*ldlr*$^{-/-}$*vldlr*$^{-/-}$ mice, followed by slow internalization, and involved heparin-releasable cell surface proteins as well as scavenger receptor BI (SR-BI). Collectively, we conclude that hepatic VLDL remnant uptake in absence of the three classical apoE-recognizing receptors is regulated by LPL activity and involves heparan sulfate proteoglycans and SR-BI.

Introduction

Lipoprotein lipase (LPL) is the key enzyme responsible for hydrolysis of triglycerides (TG) in TG-rich lipoproteins such as chylomicrons and VLDL [1, 2]. During lipolysis, the lipoproteins are reduced in size and enriched with apoE. Subsequently, their core remnants are taken up mainly by the liver via apoE-recognizing receptors, *i.e.* the LDL receptor (LDLr) and the LDLr related protein (LRP) [2]. Therefore, mice deficient for the LDLr and hepatic LRP show marked accumulation of TG-rich lipoprotein remnants [3]. Although core remnants may be directly internalized via the LDLr, the binding and internalization via the LRP is thought to involve previous binding of core remnants to heparan sulfate proteoglycans (HSPG) in the space of Disse via heparin-binding proteins such as apoE [4, 5].

The third major apoE-recognizing receptor, the VLDL receptor (VLDLr), is expressed abundantly in tissues active in fatty acid metabolism (*i.e.* heart, skeletal muscle, and white adipose tissue), and functions as a peripheral lipoprotein remnant receptor. Like the LDLr and LRP, the VLDLr binds TG-rich lipoproteins via apoE and this binding is modulated by LPL [6, 7]. VLDLr-deficient mice have normal plasma lipoprotein levels when fed a chow diet [8]. However, when TG metabolism is stressed by feeding a high fat diet or by cross-breeding on an *ob/ob* or *ldlr-/-* background, the VLDLr deficiency results in moderate accumulation of plasma TG-rich lipoproteins [9, 10]. Recently, we demonstrated that the VLDLr plays a major role in postprandial lipoprotein metabolism by facilitating LPL-mediated TG hydrolysis [11].

Additional deletion of the VLDLr from LDLr- and hepatic LRP-double deficient mice aggravates their phenotype upon stressing TG metabolism, either by high-fat feeding or by giving an intragastric olive oil bolus [12]. On a chow diet, the steady state fasted plasma TG and total cholesterol (TC) levels are 8-9-fold increased in *lrp^{-}ldlr$^{-/-}$vldlr$^{-/-}$* mice as compared to wild-type mice [12]. However, because *lrp^{-}ldlr$^{-/-}$vldlr$^{-/-}$* mice have continuous lipid input into their circulation via the production of VLDL similar to wild-type mice, the remnants in these mice must be cleared from plasma to maintain the steady state lipid levels. This indicates that, although less efficient than via the classical remnant receptors, additional pathway(s) are present for the clearance of lipoprotein remnants *in vivo*.

Two of these non-classical pathways may involve HSPG and the scavenger receptor BI (SR-BI). HSPG have been reported to directly internalize apoE-enriched TG-rich particles, both in *vitro* [13] and *in vivo* [14]. In fact, under normal physiological conditions hepatic HSPG are critically important for the clearance of remnant lipoproteins, since targeted inactivation of the biosynthetic gene *Ndst1* in hepatocytes, resulting in 50% reduction in sulfation of liver heparan sulfate, resulted in accumulation of TG-rich lipoproteins in wild-type as well as *ldlr-/-* mice [15]. In addition, recent studies using *srb1-/-* mice have shown that SR-BI can function as an internalizing receptor for chylomicrons [16, 17] and VLDL [18].

Sehayek *et al.* [19] showed that the lipolytic activity of LPL (*i.e.* hydrolysis of TG within the lipoprotein core) is a requisite for apoE-dependent uptake of lipoprotein remnants via the

LDLr and possibly the LRP *in vitro*. However, it remains unclear whether modulation of LPL activity also affects the catabolism of TG-rich particles in the absence of the three major apoE-recognizing receptors *in vivo*, *e.g.* via HSPG and/or SR-BI. Therefore, the aim of this study was to determine the role of LPL activity in hepatic VLDL metabolism in mice that lack the LDLr, hepatic LRP, and the VLDLr, as well as the mechanisms involved in the hepatic uptake. Hereto, we either increased LPL activity by adenovirus-mediated overexpression [20] or decreased LPL activity by adenovirus-mediated expression of the LPL-inhibitor apoCI [21]. In these studies, we demonstrate that the receptor-independent hepatic uptake of VLDL core remnants *in vivo* is regulated by LPL activity and involves HSPG and SR-BI.

Materials and methods

Animals

Male MX1Cre^{+}*lrploxP/loxPldlr*$^{-/-}$*vldlr*$^{-/-}$ mice [12], 4-6 months of age were used in experiments. Mice were obtained from our breeding colony at the Institutional Animal Facility and housed under standard conditions in conventional cages and were fed regular chow *ad libitum*. LRP deficiency was induced by intraperitoneal injection of polyinosinic:polycytidylic ribonucleic acid (pI:pC, Sigma, St Louis, MO, U.S.A.), which results in the complete absence of LRP protein in liver membrane extracts [12]. These mice will be further referred to as *lrp*$^{-}$*ldlr*$^{-/-}$*vldlr*$^{-/-}$. Experiments were performed after 4 h of fasting at 12:00 pm with food withdrawn at 8:00 am, unless indicated otherwise. The Institutional Ethical Committee on Animal Care and Experimentation has approved all experiments.

Adenoviruses and administration to mice

The generation of an adenovirus expressing human apoCI (Ad*APOC1*) has been described in full detail [21]. An adenovirus expressing human LPL (Ad*LPL*) [1] was a kind gift of Dr. Silvia Santamarina-Fojo. An adenovirus expressing ß-galactosidase (Ad*LacZ*) was used as control. Viruses were grown and purified by standard procedures and typical titers of $1x10^{10}$ – $1x10^{11}$ pfu/mL were obtained. Viruses were stored in aliquots at –80°C until use. Basal serum lipid levels were measured 4 weeks after pI:pC injection, at least 3 days before adenovirus injection into mice. At day 0, mice were injected into the tail vein with either Ad*APOC1*, Ad*LPL* or Ad*LacZ* ($1x10^{9}$ pfu/mouse), diluted with PBS to a total volume of 200 µL. To prevent sequestration of low doses of virus by Kupffer cells, mice were pre-injected with $0.5x10^{9}$ pfu Ad*LacZ* at 3 h before injection of the adenoviruses of interest [22].

Plasma lipid and lipoprotein analysis

In all experiments blood was collected from the tail vein into chilled paraoxon (Sigma, St. Louis, MO, U.S.A.)-coated capillary tubes to prevent ongoing *in vitro* lipolysis [23]. The tubes

were placed on ice, centrifuged at 4°C, and the obtained plasma was assayed for TC and TG, using the commercially available enzymatic kits 236691 and 11488872 (Roche Molecular Biochemicals, Indianapolis, IN, U.S.A), respectively. For determination of the distribution of lipids over plasma lipoproteins by fast-performance liquid chromatography (FPLC), 50 µL of pooled plasma per group was injected onto a Superose 6 column (Äkta System; Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.), and eluted at a flow rate of 50 µL/min with PBS, 1 mM EDTA, pH 7.4. Fractions of 50 µL were collected and assayed for TC and TG as described above.

Post-heparin plasma LPL levels

Blood was collected 10 min after tail vein injection of heparin diluted in PBS (0.1 U/g body weight; Leo Pharmaceutical Products B.V., Weesp, The Netherlands). Plasma was isolated, snap-frozen, and stored at –80°C until analysis for total LPL activity as previously described [24].

Preparation of VLDL-like emulsion particles

TG-rich VLDL-like emulsion particles (80 nm) were prepared as described [25]. Emulsions were obtained by adding the following labels to 100 mg of emulsion lipids prior to sonication: 1) 200 µCi of glycerol tri[^{3}H]oleate (triolein, TO) and 20 µCi of [^{14}C]cholesteryl oleate (CO), 2) 500 µg of 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanide perchlorate (DiI) (Molecular Probes, Leiden, The Netherlands), or 3) 80 µCi of [^{3}H]cholesteryl oleoyl ether (COEth).

In vivo kinetics of VLDL-like emulsion particles

The *in vivo* kinetics of [^{3}H]TO and [^{14}C]CO-double labeled emulsion particles were assessed at five days after virus administration. Hereto, mice were anaesthetized with acepromazine: midazolam: fentanyl (1:2:5, v/v/v), the abdominal cavities were opened, and the radiolabeled emulsion particles (1.0 mg TG in 200 µL of PBS) were injected into the vena cava inferior at 8:00 am. In addition, the effect of heparin on the kinetics of emulsion particles was evaluated. Hereto, mice were anaesthetized and the radiolabeled emulsion particles (1.0 mg TG in 200 µL of PBS) were administered into the tail vein 10 min after i.v. injection of heparin (500 U/kg) or PBS at 8:00 am. At indicated time-points after injection, blood was taken from the vena cava inferior or tail vein, to determine the serum decay of [^{3}H]TO and [^{14}C]CO by scintillation counting (Packard Instruments, Dowers Grove, IL, U.S.A.). At 30 minutes after injection, mice were sacrificed and tissues were collected, including liver, heart, skeletal muscle, and white adipose tissue (WAT). Since the various WAT compartments can have different LPL activity levels [26], we analyzed both perirenal, intestinal, and gonadal WAT. Tissues were weighed and dissolved o/n in Solvable (Packard Bioscience, Meriden, CT, U.S.A.), where after ^{3}H- and ^{14}C-activities were determined in Ultima Gold (Packard Bioscience).

Radioactivity values were corrected for the serum radioactivity (liver, 84.7 µL/g; heart, 68.1 µL/g; white adipose tissue, 16.1 µL/g; skeletal muscle, 13.7; µL/g) present at the time of sampling [25]. The total plasma volumes of the mice were calculated from the equation V (mL) = 0.04706 x body weight (g), as determined from ^{125}I-BSA clearance studies as previously described [27].

Visualisation of uptake of fluorescently labeled VLDL-like emulsion particles by isolated mouse hepatocytes

Mouse hepatocytes were isolated from anesthetized wild-type or *lrp*$^{-}$*ldlr*$^{-/-}$*vldlr*$^{-/-}$ mice and subjected to Percoll® gradient centrifugation to discard nonviable cells [28]. The cells (viability >99% as judged from 0.2% trypan blue exclusion) were attached to collagen S-coated (3.87 µg/cm^2) 2.5 cm glass cover slips in 9.6 cm^2 6-well dishes (0.8 × 10^6 cells/well) by culturing in DMEM+10% fetal calf serum (3-4 h at 37 °C). The cover slips were washed to remove unbound cells and incubated (2 h at 4°C) with DiI-labeled VLDL-like emulsion particles (100 µg TG/mL). The cover slips were washed twice with DMEM+2% BSA to remove unbound particles, and transferred to a Zeiss IM-35 inverted microscope (Oberkochen, Germany) with a Zeiss plan apochromatic 63×/1.4 NA oil objective and fitted with a temperature-controlled incubation chamber, which was equipped with a Bio-Rad 600 MRC confocal laser scanning microscopy system. The cells were further incubated (30 min at 37 °C) in DMEM+2% BSA, after which the (intra)cellular localization of DiI was visualized (λex 543 nm).

Association of radiolabeled HDL and VLDL-like emulsion particles with isolated mouse hepatocytes

Mouse hepatocytes were isolated from anesthetized wild-type or *lrp*$^{-}$*ldlr*$^{-/-}$*vldlr*$^{-/-}$ mice [28]. Cells (1-2 mg protein/mL) were incubated (3 h at 37°C) in DMEM+2% BSA with [^{3}H]COEth-labeled HDL (20 µg protein/mL) or emulsion particles (100 µg TG/mL) in the absence and presence of oxidized LDL (oxLDL; 100 µg protein/mL) or heparin (1000 U/mL) under gentle shaking in a circulating lab shaker (Adolf Kühner AG, Basel, Switzerland) at 150 rpm. After incubation, cells were pelleted by centrifugation (1 min at 50 g), and unbound radiolabeled ligands were removed by washing twice with ice-cold 50 mM Tris.HCl, 150 mM NaCl, 5 mM CaCl2 (Tris-buffered saline), pH 7.4, containing 0.2% BSA, and once with Tris-buffered saline without BSA. The cell pellet was lysed in 0.1 M NaOH and the cell-associated radioactivity and protein content was measured. [^{3}H]COEth association was calculated as dpm per mg cell protein.

Statistical analysis

All data are presented as means ± S.D. Data were analyzed using the Mann-Whitney U-test unless indicated otherwise. *P*-values less than 0.05 were regarded as statistically significant.

Results

LPL activity modulates VLDL-cholesterol levels in *lrp⁻ldlr⁻/⁻vldlr⁻/⁻* mice

To study the impact of LPL activity on the clearance of VLDL-associated TG and cholesterol in the absence of the apoE-recognizing receptors, we used mice deficient for the LDLr, hepatic LRP, and VLDLr, as previously described [12]. Upon deletion of hepatic LRP from MX1Cre⁺*lrplox/loxldlr⁻/⁻vldlr⁻/⁻* mice, their plasma lipid levels were determined and the mice were assigned to three groups, matched for TC and TG plasma levels (**Table 1**). The mice received Ad*LPL* to increase LPL levels [2], Ad*APOC1* to inhibit LPL activity [3], or Ad*LacZ* as a control group. The effect of these interventions on plasma lipid levels was assessed at 5 days after injection.

Table 1 Effect of adenovirus administration on plasma lipid levels and post-heparin LPL plasma activity in *lrp⁻ldlr⁻/⁻vldlr⁻/⁻* mice

		LPL activity (μmol FFA generated/h/mL)	TG (mM)	TC (mM)
Before adenovirus				
	Ad*LPL*	n.d.	3.9 ± 0.7	19.5 ± 3.0
	Ad*LacZ*	n.d.	4.0 ± 0.8	19.4 ± 3.3
	Ad*APOC1*	n.d.	3.8 ± 0.6	19.5 ± 8.1
After adenovirus				
	Ad*LPL*	33.7 ± 5.4a	1.1 ± 0.1b	15.7 ± 3.9a
	Ad*LacZ*	11.4 ± 1.6	7.5 ± 0.9	19.0 ± 1.9
	Ad*APOC1*	11.5 ± 7.6	37.6 ± 10.1b	30.1 ± 6.7b

Plasma was obtained from fasted *lrp-ldlr-/-vldlr-/-* mice before and after administration of Ad*LacZ*, Ad*LPL*, or Ad*APOC1*, and assayed for triglycerides (TG) and total cholesterol (TC). After the second blood withdrawal, heparin was injected and post-heparin plasma was obtained, and assayed for lipoprotein lipase (LPL) activity. Values are expressed as means ± S.D. n.d., not determined. Statistical differences were assessed as compared to *AdLacZ*. *aP*<0.05. *bP*<0.01.

Ad*LPL* administration resulted in a 3.0-fold increase in post-heparin LPL plasma activity (33.7±5.4 vs 11.4±1.6 μmol FFA generated/h/mL; *P*<0.05), with a concomitant 6.8-fold reduction in plasma TG levels (1.1±0.1 vs 7.5±0.9 mM; *P*<0.01) and 1.2-fold reduction in plasma TC levels (15.7±3.9 vs 19.0±1.9 mM; *P*<0.05) as compared to mice administered with Ad*LacZ* (Table 1). From FPLC fractionation of pooled plasma it was apparent that the decrease in plasma TG and TC was confined to the VLDL fractions (**Fig. 1**).

Ad*APOC1* administration did not result in altered total post-heparin plasma LPL levels (11.5±7.6 vs 11.4±1.6 μmol FFA/h/mL) (Table 1). This is in accordance with previous findings that human *APOC1* transgenic mice do not show a change in plasma total LPL levels as compared to wild-type mice [24], rather apoCI acts by modulating local LPL activity. Administration of Ad*APOC1* resulted in 5.0-fold increased plasma TG levels (37.6±10.1 vs 7.5±0.9 mM; *P*<0.01) in addition to 1.6-fold increased plasma TC levels (30.1±6.7 vs 19.0±1.9 mM;

P<0.01) as compared to Ad*LacZ* control mice (Table 1). These increased plasma TG and TC levels were due to increased VLDL levels, as was shown after FPLC fractionation of pooled plasma (Fig. 1).

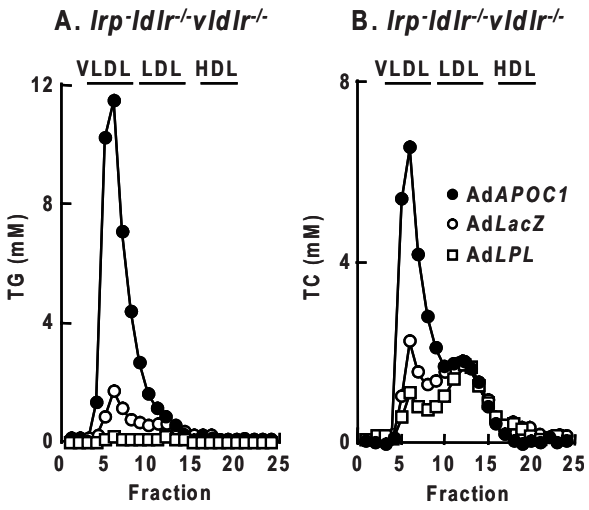

Figure 1 Effect of modulation of LPL activity by LPL and apoCI on plasma triglyceride and total cholesterol distribution.

Lrp-ldlr-/-vldlr-/- mice were injected with AdLacZ (open circles), AdLPL (open squares), or AdAPOC1 (closed circles) (1x109 pfu). 4 Days after injection 4h fasted plasma samples were drawn, pooled, and subjected to FPLC fractionation. Fractions were analyzed for triglycerides (TG) (A) and total cholesterol (TC) (B).

Taken together, these results suggest that LPL activity not only regulates the clearance of VLDL-TG, but also determines the clearance of VLDL-cholesterol in absence of the LRP, LDLr, and VLDLr *in vivo*.

LPL activity modulates the liver association of VLDL-like emulsion core remnants in *lrp-ldlr-/-vldlr-/-* mice

To provide direct *in vivo* evidence that LPL activity determines the clearance of VLDL-cholesterol, [^{3}H]TO and [^{14}C]CO double-labeled TG-rich VLDL-like emulsion particles were injected into *lrp-ldlr-/-vldlr-/-* mice 5 days after Ad*LPL*, Ad*APOC1*, or Ad*LacZ* administration (**Fig. 2**).

The clearance of [^{3}H]TO was substantially accelerated in Ad*LPL* treated mice as compared to control mice, evidenced by a 2.5-fold decreased serum half-life of ^{3}H-activity (t1/2=18±7 vs 45±11 min) (Fig. 2A). On the other hand, mice that were treated with Ad*APOC1* showed a 1.7-fold increased half-life (t1/2=77±13). Thus, the LPL activity was positively correlated with the serum clearance of ^{3}H-activity (*P*<0.05). Increased LPL activity was accompanied by a significantly increased uptake of TO-derived ^{3}H-activity in LPL-expressing organs as

heart (P<0.01), muscle (P<0.05), and WAT (P<0.05 for perirenal WAT), and also in the liver (P<0.01) (Fig. 2B).

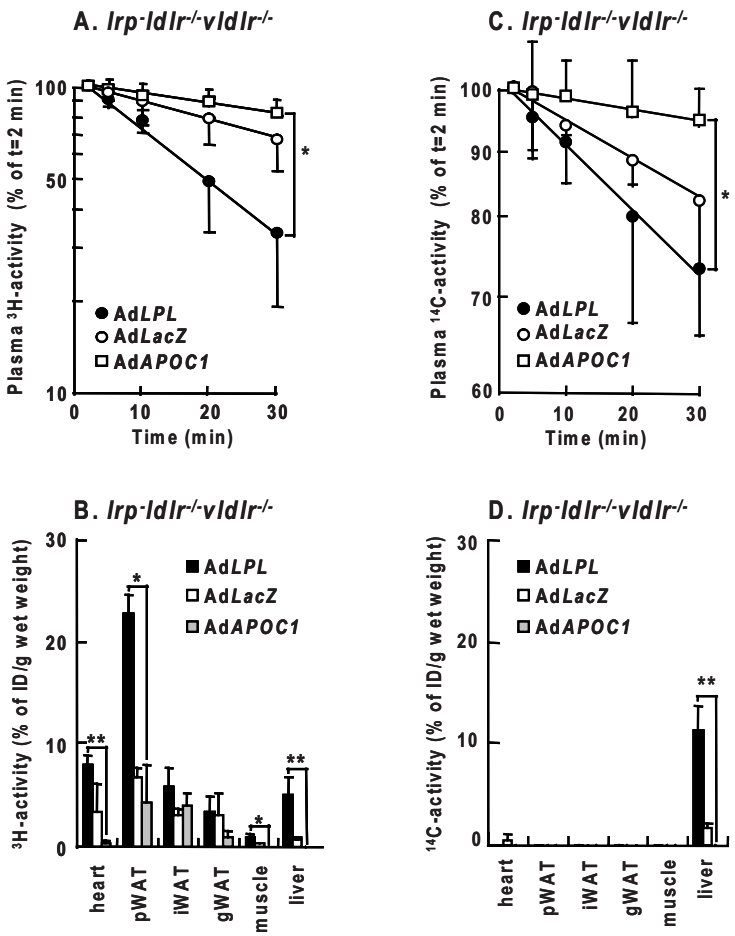

Figure 2 Effect of modulation of LPL activity by LPL and apoCI on the serum decay and organ distribution of VLDL-like emulsion particles.

Lrp-ldlr-/-vldlr-/- mice were injected with AdLacZ, AdLPL, or AdAPOC1 (1x109 pfu). 5 Days after injection mice were anaesthetized and injected with [3H]TO and [14C]CO double-labeled emulsion particles (1 mg TG). Serum samples were collected at indicated times and measured for 3H-activity (A) and 14C-activity (C). At t=30 min the animals were sacrificed and tissues collected. Tissues were dissolved in Solvable (o/n at 60°C) and measured for 3H-activity (B) and 14C-activity (D), corrected for serum radioactivity. Asterisks indicate a statistically significant difference (*P<0.05; **P<0.01) as analyzed by one-way ANOVA (A, C) or Mann-Whitney U-test (B, D). pWAT, perirenal white adipose tissue (WAT); iWAT, intestinal WAT; gWAT, gonadal WAT.

Although serum [^{14}C]CO decay was slower as compared to the [^{3}H]TO decay, the [^{14}C]CO serum half-life was also dependent on LPL activity. This was evidenced by a 1.4-fold decreased half-life of ^{14}C-label in Ad*LPL* treated mice (t1/2=71±24 min) and a 3.7-fold increased half-life in Ad*APOC1* treated animals (t1/2= 364±241 min) as compared to controls (t1/2= 99±8 min) (P<0.05) (Fig. 2C). In addition, the association of ^{14}C-activity with the liver was 7.0-fold increased in Ad*LPL* animals, as compared to control mice, and decreased to zero by Ad*APOC1* (P<0.01) (Fig. 2D). Further analysis of the distribution of ^{14}C-activity over cholesterol and cholesteryl esters in the liver revealed that the ^{14}C-activity was almost exclusively recovered in the cholesteryl esters (data not shown), which indicates that particles are indeed associated with the liver, however, they have not entered the lysosomal degradation pathway yet. Taken together, these results show that LPL activity positively correlates with liver association of particle core remnants.

Heparin accelerates the lipolysis of VLDL-like emulsion particles followed by rapid liver association of their core remnants in wild-type and *lrp*$^{-}$*ldlr*$^{-/-}$*vldlr*$^{-/-}$ mice

To gain insight into the potential pathways by which VLDL-like emulsion particles associate with the liver in absence of the LDLr, hepatic LRP, and VLDLr, we next determined the kinetics of [^{3}H]TO and [^{14}C]CO double-labeled TG-rich VLDL-like emulsion particles in wild-type mice and *lrp*$^{-}$*ldlr*$^{-/-}$*vldlr*$^{-/-}$ mice following an i.v. injection of heparin (**Fig. 3**). Heparin results in the release of LPL from the vascular endothelium, resulting in a higher systemic plasma TG hydrolase activity, and prevents the association of both LPL and hepatic lipase (HL) with the liver.

In wild-type mice, heparin accelerated the serum clearance of both [^{3}H]TO (t1/2 1.3±0.1 min vs 8.7±1.6 min) and [^{14}C]CO (t1/2 2.9±0.1 min vs 12.0±2.4 min) (not shown). This was accompanied by a decreased uptake of [^{3}H]TO-derived radiolabel by peripheral tissues and an increased uptake by the liver (Fig. 3A). [^{14}C]CO-derived radiolabel mainly associated with the liver, both in the absence (35.0±5.1% of ID/g) and presence (41.3±7.0% ID/g) of heparin (Fig. 3B).

In *lrp*$^{-}$*ldlr*$^{-/-}$*vldlr*$^{-/-}$ mice, heparin also accelerated the serum clearance of both [^{3}H]TO (t½ 1.7±0.3 min vs 2.9±0.7 min) and [^{14}C]CO (t½ 2.8±0.8 min vs 6.4±2.0 min) (not shown). The serum decay of the VLDL-like emulsion particles in *lrp*$^{-}$*ldlr*$^{-/-}$*vldlr*$^{-/-}$ mice was faster than observed in the previous experiment. This was probably due to the lower TG levels in this specific batch of mice at the time of the experiment (2.2±1.0 mM vs 4.0±0.8 mM) and the fact that infection of mice with a recombinant adenovirus *per se* did affect TG levels to some extent in the previous experiment (7.5±0.9 mM vs 4.0±0.8 mM). Similarly to wild-type mice, the increased lipolysis after heparin injection was accompanied by an increased association of [^{3}H]TO-derived radiolabel with the liver (Fig. 3C). Again similarly to wild-type mice, heparin did not affect the liver association of the core remnants in *lrp*$^{-}$*ldlr*$^{-/-}$*vldlr*$^{-/-}$ mice, as

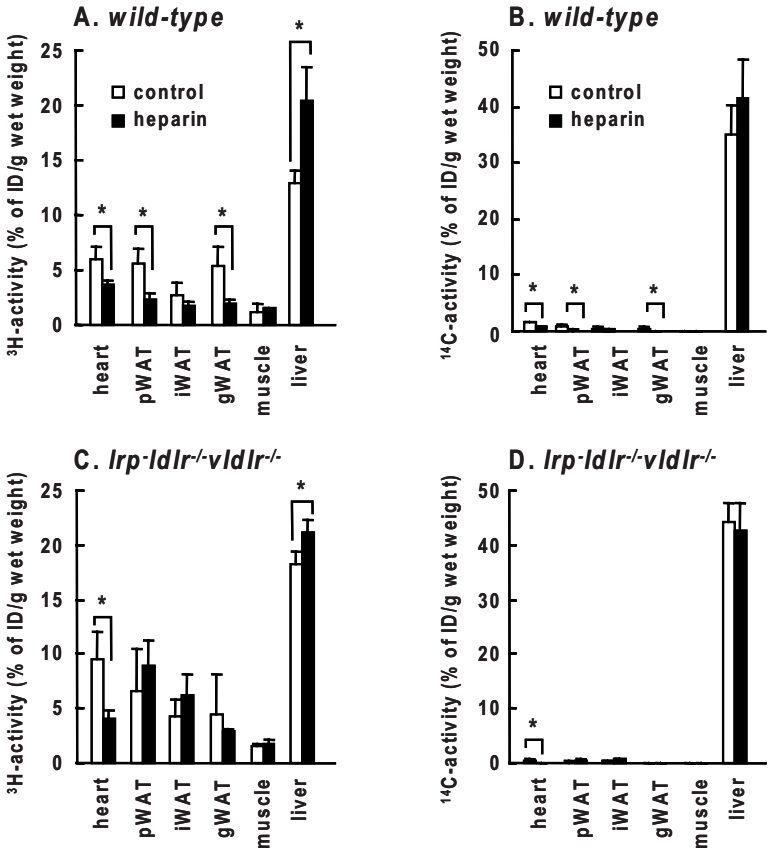

Figure 3 Effect of heparin on the organ distribution of VLDL-like emulsion particles.
Wild-type mice (A, B) and lrp-ldlr-/-vldlr-/- mice (C, D) were anaesthetized and injected with 500 U/kg heparin (closed bars) or vehicle (open bars). After 10 min, mice were injected with [3H]TO and [14C]CO double-labeled emulsion particles (1 mg TG). At t=30 min the animals were sacrificed and tissues collected. Tissues were dissolved in Solvable (o/n at 60°C) and measured for 3H-activity (A, C) and 14C-activity (B, D), corrected for serum radioactivity. Asterisks indicate a statistically significant difference (*P<0.05).

judged from an effective liver association of [^{14}C]CO both in the absence (44.3±3.5% of ID/g) and presence (42.6±5.0% ID/g) of heparin (Fig. 3D). Since heparin releases LPL and HL from endothelial surfaces and the liver, the effective liver association of particle core remnants in *lrp^{-}ldlr$^{-/-}$vldlr$^{-/-}$* mice apparently does not depend on the bridging function of LPL and HL.

Association of VLDL-like emulsion particles with hepatocytes from *lrp*$^{-}$*ldlr*$^{-/-}$*vldlr*$^{-/-}$ mice is followed by slow internalization

We have previously shown that the hepatic binding and uptake of TG-rich lipoproteins and VLDL-like emulsion particles is mainly exerted by hepatocytes [29]. To establish whether binding of VLDL-like emulsion particles to hepatocytes that lack the LDLr, LRP, and VLDLr, can still lead to internalization, we incubated freshly isolated hepatocytes with DiI-labeled emulsion particles (**Fig. 4**). These emulsion particles avidly bound to the cell surface upon incubation at 4°C. LDLr- and LRP-independent internalization of cell-bound emulsion particles was observed on further incubation at 37°C, as evidenced by the detection of fluorescence in compartments below the cell surface after 30 min of incubation (Fig. 4A). However, the rate of internalization was slower as compared to that of wild-type hepatocytes, which internalized the majority of cell-associated particles within the same time period (Fig. 4B).

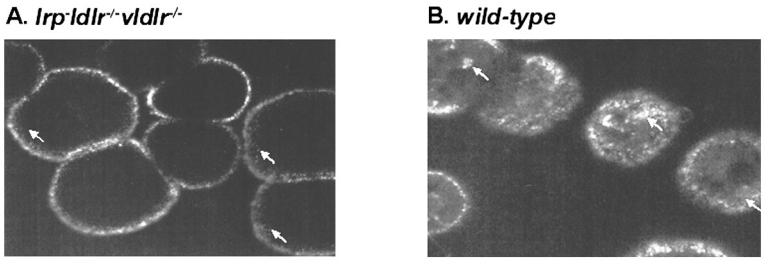

Figure 4 **Effect of LRP and (V)LDL receptor-deficiency on the uptake of VLDL-like emulsion particles by hepatocytes.** Freshly isolated hepatocytes from lrp-ldlr-/-vldlr-/- mice (A) and wild-type mice (B) were incubated (2 h at 4°C) in DMEM+2% BSA with 50 nm sized DiI-labeled emulsion particles (100 µg TG/mL). The cells were washed to remove unbound particles and further incubated at 37°C. After 30 min, localization of DiI was determined by confocal laser scanning microscopy. Intracellular fluorescently labeled compartments are indicated by arrows. Under the applied conditions, autofluorescence was negligible.

Association of VLDL-like emulsion particles with hepatocytes from *lrp*$^{-}$*ldlr*$^{-/-}$*vldlr*$^{-/-}$ mice involves HSPG-bound ligands and SR-BI

Since both cell surface HSPG [15] and SR-BI [18] have recently been implicated in the hepatic uptake of VLDL, we examined their contribution to the association of [^{3}H]COEth-labeled VLDL-like emulsion particles with freshly isolated hepatocytes from wild-type mice versus *lrp*$^{-}$*ldlr*$^{-/-}$*vldlr*$^{-/-}$ mice after incubation for 3 h at 37°C. Deficiency for the LRP, LDLr and VLDLr did upregulate SR-BI expression to some extent, since the association of [^{3}H]COEth-HDL with hepatocytes from *lrp*$^{-}$*ldlr*$^{-/-}$*vldlr*$^{-/-}$ mice was higher than with hepatocytes from wild-type mice (+26%; $P<0.05$) (**Fig. 5**). The established SR-BI inhibitor oxLDL [30] inhibited the association of HDL with both cell types (approx. -60%; $P<0.001$).

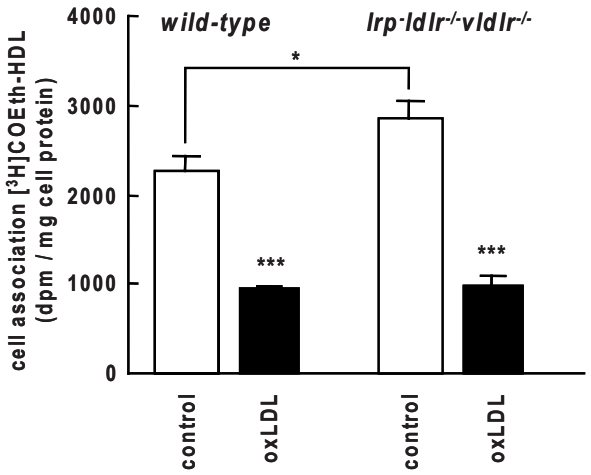

Figure 5 **Effect of LRP and (V)LDL receptor-deficiency on the association of HDL with hepatocytes.** Freshly isolated hepatocytes from wild-type mice (left) and lrp-ldlr-/-vldlr-/- mice (right) were incubated (3 h at 37°C) in DMEM+2% BSA with [3H]COEth-labeled HDL (20 µg of protein/mL) in absence or presence of oxLDL (100 µg protein/mL). The cells were washed to remove unbound particles and the cell association of [3H]COEth was measured as dpm/mg cell protein. Values are means ± SD (n=3). Asterisks indicate a statistically significant difference (*P<0.05; ***P<0.001).

Remarkably, the cell association of [^{3}H]COEth-emulsion particles was unaffected by deficiency for the LRP, LDLr and the VLDLr (**Fig. 6**), confirming the contribution of pathways other than these classical apoE-receptors to the association of emulsion particles with hepatocytes. Heparin, at a concentration of 1000 U/mL that releases all heparin-binding proteins from cell surface HSPG including apolipoproteins from cell receptors [13], strongly inhibited the cell association (>95%). Also, oxLDL effectively inhibited the association of [^{3}H]COEth-emulsion particles with wild-type (-60%) and *lrp-ldlr$^{-/-}$vldlr$^{-/-}$* hepatocytes (-78%) to a similar extent as [^{3}H]COEth-HDL (approx. 60%), indicating the involvement of SR-BI in the cell association of emulsion particles. To evaluate the effect of the bridging function of LPL and apoE, we also determined the cell association of emulsion particles after previous enrichment with heat-inactivated LPL and apoE. Both LPL and apoE reduced the association of emulsion particles with wild-type and *lrp-ldlr$^{-/-}$vldlr$^{-/-}$* hepatocytes. However, both heparin and oxLDL still inhibited the association of these LPL- and apoE-containing emulsion particles with both types of hepatocytes (Fig. 6).

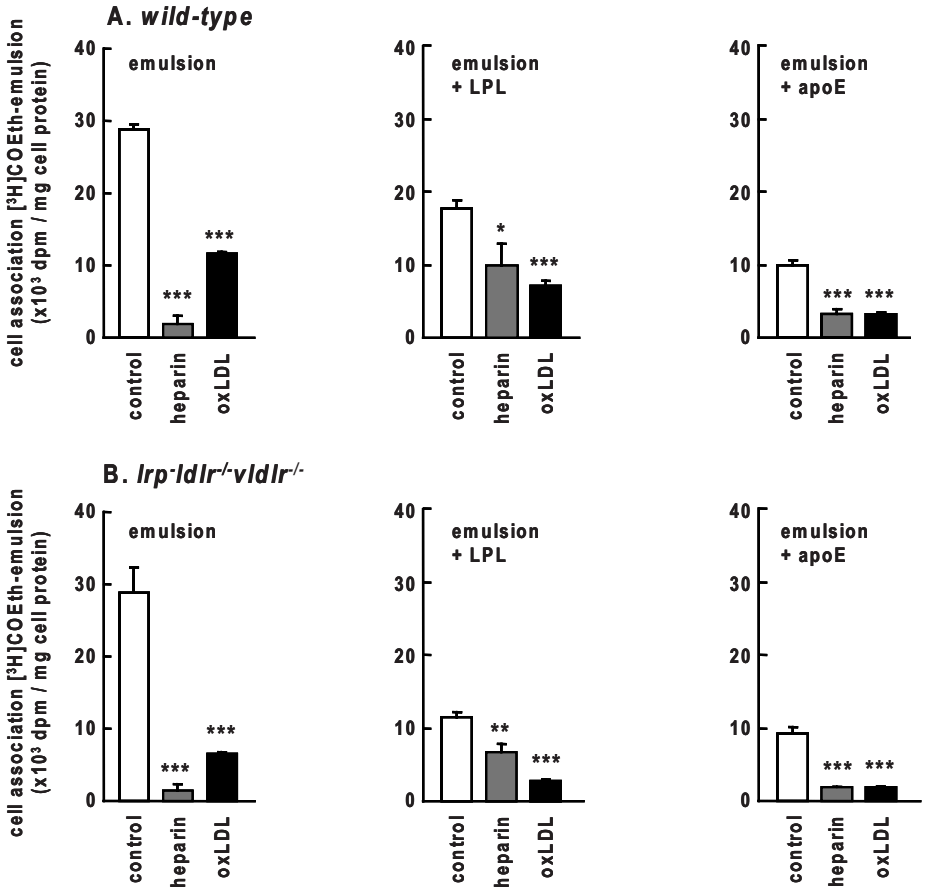

Figure 6 Effect of LRP and (V)LDL receptor-deficiency on the association of VLDL-like emulsion particles with hepatocytes.

Freshly isolated hepatocytes from wild-type mice (A) and lrp-ldlr-/-vldlr-/- mice (B) were incubated (3 h at 37°C) in DMEM+2% BSA with 50 nm sized [3H]COEth-labeled emulsion particles (100 µg TG/mL), without or with previous enrichment (30 min at 37°C) with heat-inactivated LPL (1 µg/mL) or human apoE (6 µg/mL), in absence or presence of heparin (1000 U/mL) or oxLDL (100 µg protein/mL). The cells were washed to remove unbound particles and the association of [3H]COEth was measured as dpm/ mg cell protein. Values are means ± SD (n=3). Asterisks indicate a statistically significant difference (*P<0.05; **P<0.01; ***P<0.001).

Discussion

The aim of the current study was to investigate the role of LPL activity in the classical apoE-receptor-independent clearance of lipoprotein remnants, by modulating LPL activity in *lrp-ldlr$^{-/-}$vldlr$^{-/-}$* mice, and to determine the mechanisms involved in their hepatic uptake. We demonstrate that the hepatic uptake of VLDL core remnants independent of these three main apoE-recognizing receptors is positively regulated by LPL activity, and involves HSPG and SR-BI.

How could modulation of LPL activity affect the hepatic uptake of lipoprotein remnants in absence of the LDLr, hepatic LRP, and VLDLr? *In vitro* studies have shown that LPL via its bridging function can mediate the binding and uptake of lipoproteins and their remnants via HSPG [31-34], LRP [35], and the LDLr [34, 36]. A function of catalytically inactive LPL in the hepatic lipoprotein uptake has also been demonstrated *in vivo* [37, 38]. Likewise, HL has been demonstrated to participate in the cell binding and uptake of remnant lipoproteins *in vitro* [39, 40] and mediate the clearance of remnant lipoproteins from plasma by its non-lipolytic bridging function *in vivo* [41]. However, heparin, which effectively releases LPL and HL from cellular binding sites and results in increased lipolysis by enhancing the interaction of the catalytically active lipases with lipoproteins, did not abrogate the rapid binding of VLDL-like emulsion-associated [^{14}C]CO activity with the liver in either wild-type and *lrp^{-}ldlr$^{-/-}$vldlr$^{-/-}$* mice. Furthermore, the *in vitro* studies with isolated hepatocytes showed that enrichment of VLDL-like emulsion particles with catalytically inactive LPL decreased rather than increased the cell association of the particles. These data indicate that the rapid initial hepatic association of the particle core remnants in *lrp^{-}ldlr$^{-/-}$vldlr$^{-/-}$* mice is likely to be independent of the bridging function of LPL and HL. This conclusion is corroborated by our observation that Ad*APOC1* reduced the hepatic association of the particle core remnants without affecting either post-heparin plasma levels of LPL activity (11.5±7.6 vs 11.4±1.6 µmol FFA/h/mL) or LPL protein (295±40 vs 254±53 ng/mL) as compared to Ad*LacZ*. Taken together, the positive correlation between plasma LPL activity and hepatic VLDL remnant uptake in *lrp^{-}ldlr$^{-/-}$vldlr$^{-/-}$* mice does not seem to be caused by the bridging functions of LPL and HL. Most likely, it is a consequence of the catalytic function of LPL, which accelerates the generation of VLDL core remnants that subsequently become associated with the liver via mechanisms unrelated to the classical apoE receptors and lipases.

So what mechanisms do underlie the binding and uptake of VLDL remnants by the liver through apparently non-classical pathways? Although it has been suggested that LRP5 [42], apoB-48 receptor [43] and LR11 [44] may play a role in the metabolism of apoB-containing lipoproteins, the most likely candidates appear to be HSPG [15] and SR-BI [16, 18].

It has previously been shown that lactoferrin inhibits the binding of chylomicron remnants and ß-VLDL to HSPG *in vitro* [45], since lactoferrin effectively binds to HSPG probably due to its structural homology with the heparin binding site of apoE and its overall positive charge [46]. In addition, lactoferrin reduces the hepatic association of the presently applied VLDL-like

emulsion particles by 90% *in vivo* [25], indicating that hepatic association of these particles is dependent on HSPG. Protamine, like lactoferrin, binds to HSPG by electrostatic interaction, thereby reducing remnant binding [47]. We have shown that protamine administration to mice that lack both the LDLr and hepatic LRP completely inhibited the liver association of the emulsion particles (unpublished observations by P.C.N.R.). These observations thus underscore the involvement of HSPG in the hepatic association of VLDL-like emulsion particle remnants. Indeed, treatment of hepatocytes from wild-type mice and *lrp*$^{-}$*ldlr*$^{-/-}$*vldlr*$^{-/-}$ mice with heparin at a concentration that releases a number of ligands from cell surface HSPG including apolipoproteins from cell receptors [48, 49], strongly inhibited the cell association (>95%). This indicates the involvement of HSPG-bound ligands in the binding and possibly also uptake of VLDL-like emulsion particles by hepatocytes.

Our observation that heparin did not reduce the liver association of the VLDL-like particle core remnants in wild-type and *lrp*$^{-}$*ldlr*$^{-/-}$*vldlr*$^{-/-}$ mice is in accordance with previous observations in *ldlr-/-* mice [50]. The seeming discrepancy that heparin blocks the association of emulsion particles with hepatocytes *in vitro*, but not with the liver *in vivo*, is probably related to the additional effect of heparin *in vivo* (*i.e.* accelerated remnant formation through increasing plasma LPL activity) as well as to the difference between the doses of heparin used in the *in vitro* and *in vivo* studies. Al-Haideri et al. [13] have shown that a high concentration of 1000 U/mL releases all heparin-binding proteins from cells, thereby blocking the association of similar emulsion particles to fibroblasts, whereas a low concentration of 10 U/mL only displaces a number of ligands from HSPG and had no effect on uptake of emulsion particles by fibroblasts. Accordingly, we observed that 1000 U/mL heparin blocked association of emulsion particles with hepatocytes *in vitro*, and the generally applied dosage of 500 U/kg heparin (which corresponds to 10-15 U/mL plasma) did not reduce the liver binding of emulsion particles *in vivo*. Taken together with the fact that heparin at a concentration of 100 U/mL only marginally affects the binding of newly secreted apoE to cells [5] and does not release apoE from immobilized HSPG [51], it is well possible that under the *in vivo* conditions hepatocytes still contain sufficient apoE on the cell surface to allow sequestration of remnants on the hepatocyte surface, and to facilitate remnant uptake via its secretion-capture role [5]. In addition, it has been shown that the VLDL-like emulsion particles that we used in this study rapidly acquire apoE from plasma [25, 52], which would allow plasma-derived apoE to also play a role in the subsequent uptake of emulsion particles by hepatocytes.

SR-BI has recently been identified as a receptor that internalizes VLDL holoparticles [18]. Our *in vitro* data showed that SR-BI expression by hepatocytes was functionally upregulated to some extent by the deficiency for the LDLr, LRP and VLDLr. The established SR-BI inhibitor oxLDL [30] markedly reduced the association of [^{3}H]COEth-labeled VLDL-like emulsion particles with hepatocytes from both wild-type and *lrp*$^{-}$*ldlr*$^{-/-}$*vldlr*$^{-/-}$ mice to a similar extent as that of [^{3}H]COEth-labeled HDL, which indeed indicates the involvement of SR-BI in the binding and uptake of VLDL-like emulsion particles by hepatocytes. SR-BI recognizes an

array of ligands, including apolipoproteins and negatively charged phospholipids. Since the phospholipid surface of the emulsion particles has a negligible charge, as evident from a low electrophoretic mobility on agarose gel as compared to (oxidized) lipoproteins [25], it is unlikely that SR-BI directly binds to the emulsion-associated phospholipids. Instead, the association of VLDL core remnants with hepatocytes through SR-BI most likely involves apoE. Mice deficient for the LDLr have elevated plasma apoE levels as compared to wild-type mice, which are maintained upon the deletion of LRP [3, 53] and the VLDLr [12], and apoE is a well-established ligand for SR-BI [54-56].

In conclusion, we have demonstrated that in the absence of the three major apoE-recognizing receptors, the uptake of VLDL remnants by the liver is regulated by the catalytic function of LPL in plasma and involves both hepatic HSPG and SR-BI.

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Acknowledgments

We thank J. Kar Kruijt (Div. Biopharmaceutics, LACR, Leiden) for excellent technical assistance. This work was performed in the framework of the Leiden Center for Cardiovascular research LUMC-TNO, and supported by the Leiden University Medical Center (Gisela Thier Fellowship to P.C.N.R.), The Netherlands Organization for Scientific Research (NWO grant 908-02-097 and NWO VIDI grant 917.36.351 to P.C.N.R.) and The Netherlands Heart Foundation (NHS grant 2003B136 to P.C.N.R.).

Chapter 5

Macrophage low-density lipoprotein receptor-related protein deficiency enhances atherosclerosis in ApoE/LDLR double knockout mice.

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Arterioscler Thromb Vasc Biol (2006) 26:2710-5

Abstract

Objective – *In vitro* studies implicate that the low-density lipoprotein receptor (LDLR)-related protein (LRP) in macrophages has a pro-atherogenic potential. In the present study, we investigated the *in vivo* role of macrophage specific LRP in atherogenesis independent of its role in the uptake of lipoproteins.

Methods and Results – We generated macrophage specific LRP deficient mice on an ApoE/LDLR double deficient background. Macrophage LRP deletion did not affect plasma cholesterol and triglyceride levels, lipoprotein distribution, and blood monocyte counts. Nevertheless, macrophage LRP deficiency resulted in a 1.8-fold increase in total atherosclerotic lesion area in the aortic root of 18-week old mice. Moreover, LRP deficiency also resulted in a relatively higher number of advanced lesions. Whereas macrophage and smooth muscle cell content did not differ between LRP deficient mice and control littermates, a 1.7-fold increase in collagen content and 2.3-fold decrease in relative number of CD3+ T cells were observed in lesions from macrophage specific LRP deficient mice.

Conclusions – Our data demonstrate that, independent of its role in lipoprotein uptake, absence of LRP in macrophages resulted in more advanced atherosclerosis and in lesions that contained more collagen and less CD3+ T cells. In contrast to previous *in vitro* studies, we conclude that macrophage LRP has an atheroprotective potential and may modulate the extracellular matrix in the atherosclerotic lesions.

Introduction

Cardiovascular diseases are the leading cause of morbidity and mortality in the Western World. The primary cause of cardiovascular diseases is atherosclerosis, which is characterised by lipid accumulation and inflammation in the vascular wall.[1,2] Macrophages play a central role in the pathogenesis of atherosclerosis by internalising modified low-density lipoprotein (LDL), production of cytokines and growth factors, and thus stimulate migration and proliferation of smooth muscle cells (SMCs), and plaque development and progression.[1]

The LDL receptor (LDLR)-related protein (LRP) is a large cell-surface multi-ligand endocytic clearance and signalling receptor of the LDLR gene family.[3-8] LRP is known to recognise >50 structurally and functionally different ligands.[9,10] It is expressed in a variety of cell types including hepatocytes, SMCs, and macrophages.[11] The hepatic LRP was originally identified as an endocytic receptor for apolipoprotein E (apoE)-rich lipoproteins.[4] Recently, we showed that hepatic LRP deficiency in mice increased atherosclerosis independent of plasma lipoproteins.[12] Similarly, SMC specific LRP deficient mice display impaired vessel wall integrity and have increased susceptibility to cholesterol-diet induced atherosclerosis.[7] These data show that LRP protects against the development of atherosclerosis at the level of the liver and the SMC, independent of its role in the removal of plasma lipoproteins.

In contrast, several lines of *in vitro* evidence show that LRP in macrophages has pro-atherogenic properties. First, LRP is highly expressed in atherosclerotic lesions and upregulated in macrophages undergoing foam cell formation.[13,14] Second, LRP regulates β2-integrin-mediated adhesion of monocytes to endothelial cells,[15] allowing monocytes to migrate into the intima and to differentiate into macrophages. Third, macrophage LRP has also been demonstrated to play a role in the translocation of 12/15-lipoxygenase, which stimulates the formation of oxidised LDL.[16,17] Finally, in concert with the LDLR, LRP can mediate the uptake of apoE-rich atherogenic lipoproteins into the macrophage.[18-20] Because all these processes promote the formation of foam cells, one would predict that LRP promotes the development of atherosclerosis at the level of macrophages.

In the present study, we investigated the role of macrophage LRP in the development of atherosclerosis *in vivo*. To this end, macrophage LRP was constitutively deleted in macrophages, using the lysozyme M Cre/loxP system.[21] Because apoE LDLR double deficient mice develop spontaneously human-like atherosclerosis without the necessity of a cholesterol-rich diet, macrophage LRP was deleted on an apoE[22] and LDLR[23] double deficient background. Moreover, this model allowed us to study the role of macrophage LRP independent of its classical role in the uptake of lipoproteins via the apoE- and LDLR-mediated pathway. Our data demonstrate that the absence of macrophage LRP results in more advanced atherosclerosis, and in lesions that contain more collagen and less CD3+ T cells. In contrast to what *in vitro* studies would predict, we conclude that, like LRP on hepatocytes and SMCs, macrophage LRP has an atheroprotective potential.

Materials and Methods

Mice

Mice with or without Cre recombinase under the lysozyme M promoter (kindly provided by I. Förster, University of Munich, Germany) were crossbred with our previously generated $LRP^{flox/fox}$ [4] $apoE^{-/-}$ [22] $LDLR^{-/-}$ [23] to generate mice either with lysozymal M Cre[21] ($LysMCre^{+}$ $LRP^{flox/flox}$ $apoE^{-/-}$ $LDLR^{-/-}$ further referred to as mϕ LRP-) or without lysozymal M Cre ($LRP^{flox/flox}$ $apoE^{-/-}$ $LDLR^{-/-}$ further referred to as mϕ LRP+).[12] Mice were genotyped by polymerase chain reaction (PCR) for LysMCre, $LRP^{flox/flox}$, apoE and LDLR status.[4,21-23] After two breeding rounds we obtained mice with the $LysMCre^{+}$ $LRP^{flox/+}$ $apoE^{-/-}$ $LDLR^{-/-}$ genotype. LRP and lysozyme M are both localised on chromosome 10 in mice. Therefore, our third breeding ($LysMCre^{+}$ $LRP^{flox/+}$ $apoE^{-/-}$ $LDLR^{-/-}$ x $LRP^{flox/flox}$ $apoE^{-/-}$ $LDLR^{-/-}$) generated only 2 out of 63 (3%) mice that were $LysMCre^{+}$ $LRP^{flox/flox}$ $apoE^{-/-}$ $LDLR^{-/-}$, instead of 15 out of 63 (25%) as expected in case of a Mendelian inheritance. These two mice were subsequently used for generating $LysMCre^{+}$ $LRP^{flox/flox}$ $apoE^{-/-}$ $LDLR^{-/-}$ mice in the consecutive breedings. The inheritance pattern of the offsprings was Mendelian (19 female mice with the $LysMCre^{+}$ $LRP^{flox/flox}$ $apoE^{-/-}$ $LDLR^{-/-}$ genotype vs. 18 female mice with the $LRP^{flox/flox}$ $apoE^{-/-}$ $LDLR^{-/-}$ genotype). Subsequently, these mice were used for our experimental breeding yielding more female mice with the $LRP^{flox/flox}$ $apoE^{-/-}$ $LDLR^{-/-}$ genotype (n = 31) than the $LysMCre^{+}$ $LRP^{flox/flox}$ $apoE^{-/-}$ $LDLR^{-/-}$ genotype (n = 17). A similar pattern was observed for male mice (18 mice with $LysMCre^{+}$ $LRP^{flox/flox}$ $apoE^{-/-}$ $LDLR^{-/-}$ genotype and 24 mice with $LRP^{flox/flox}$ $apoE^{-/-}$ $LDLR^{-/-}$ genotype). Mice were fed a SDS standard rodent chow diet (Technilab-BMI, Someren, The Netherlands) and water ad libitum. All animal experiments were approved by the institutional committee on animal welfare of TNO Quality of Life.

Macrophage LRP deletion

Peritoneal macrophages were obtained from mϕ LRP+ and mϕ LRP- mice four days after intraperitoneal injection of 1 ml thioglycollate broth (3% w/vol, Difco Grayson, GA) by flushing the peritoneum with 10 ml ice-cold phosphate buffered saline (PBS). Macrophages were washed twice with ice-old PBS and subsequently incubated in RPMI 1640 containing 10% foetal calf serum and streptomycine/penicilline in 12 cm culture plates at 37°C for 4 hours. After 3 washes with warm PBS, macrophages were lysed with 4 ml lysis buffer (0.1 M Tris, 1 M EDTA, 0.2 M NaCl, 0.002% SDS, pH 8.6) containing 1 mg proteinase K at 55°C for 30 minutes. DNA was then isolated using the standard phenol/chloroform/iso-amylalcohol method.[24] The LysMCre/loxP mediated recombination of the conditional LRP allele in the macrophages was established by PCR amplification of primer LRP postlox (5'-GTA GTT ATT CGG ATC ATC AGC TA-3', Invitrogen, The Netherlands) and mLRP12 (5'-GGT GTG ACA TAG AGT TTT AAA GAG G-3'), yielding a 400-base pair recombination product.[4]

Blood sampling and analysis

Blood samples were obtained via tail bleeding. Samples were collected in EDTA-coated vials (Sarstedt, Nümbrecht, Germany). Plasma cholesterol levels were measured by a commercially available enzymatic kit (Roche Diagnostics GmbH, Mannheim, Germany). Plasma triglyceride levels were measured by a commercially available one-step enzymatic kit that detects free and triglyceride-derived glycerol levels (Roche Diagnostics GmbH, Mannheim, Germany). Plasma lipoproteins were size-fractionated by fast protein liquid chromatography (FPLC) followed by the determination of the cholesterol levels in the individual fractions. Total blood leukocyte ($CD45^+$), T cell ($CD3^+$), B cell ($CD19^+$) and monocyte/granulocyte ($CD11b^+$) numbers were determined by fluorescence activated cell sorting (FACS) analysis (TruCOUNT, FACSalibur, BD Biosciences, CA), as described previously.[25]

Atherosclerosis analysis

Female mϕ LRP- mice and control mϕ LRP+ littermates were sacrificed at 18-week of age (n = 17 and n = 31 for mϕ LRP- and mϕ LRP+, respectively). Heart and aorta were perfused with PBS and were subsequently fixed in phosphate-buffered 4% formaldehyde (pH 7.4), dehydrated overnight and embedded in paraffin. Hearts were cross-sectioned (5 µm) with 40-µm intervals throughout the entire aortic root. Sections of the aortic valve area were routinely stained with hematoxylin-phloxine-saffran for morphometric analysis and characterisation of the lesions. For each mouse, 3 lesions at the aortic root were analysed. Per mouse, 4 cross-sections were used for quantification of atherosclerotic lesion area.[26] Areas were determined using Leica Qwin image software (EIS, Asbury, NJ). Atherosclerotic lesions were classified according to severity (*i.e.* early, moderate or advanced lesions), as described previously.[27] The numbers observed in each category were expressed as percentage of the total number of lesions observed within one group of mice.[27]

Lesion composition analysis

Serial sections of the aortic valve area were stained with rabbit anti-mouse macrophage antibody (AIA-312040, 1/1500, Accurate Chemical and Scientific, Westbury, NY) and a monoclonal mouse anti-smooth muscle cell α-actin antibody (clone 1A4, M-851, 1/1600, DakoCytomation, Belgium), as described previously.[12] Serial sections of the aortic valve area were stained with goat α-human matrix metalloproteinase-9 (MMP-9) antibody (C-20, Santa Cruz) or with rat α-human CD3 (Serotec). Sections were deparaffinised. Endogenous peroxidase was quenched with 0.3% H2O2 in 100% methanol, and nonspecific binding was blocked with 5% BSA in PBS. Antigen was retrieved by 0.1% trypsin (w/v) in PBS at 37°C for 30 minutes prior to MMP-9 staining or heat treatment in 1 mM EDTA, pH 8.0 for 20 minutes prior to CD3 staining. The primary MMP-9 (1:200) or CD3 antibody (1:1000) in 1% BSA in PBS was incubated o/n at 4°C followed by a 1-hour incubation with biotinylated secondary rabbit anti-goat (1:300) or goat α-rat antibody in 1% BSA in PBS. Following incubation with horseradish per-

oxidase labeled avidin-biotin complex (abc/HRP) (DAKO), peroxidase activity was visualised using NovaRED (Vector). Sections were counterstained with Mayer's hematoxylin.
Collagen was stained using Sirius red (Chroma-Gesellschaft, Stuttgart, Germany). Lesion macrophage (AIA-31240–positive area), smooth muscle cell (α-actin-positive area), MMP-9 and collagen (Sirius red-positive area) areas were quantified using EIS. Number of CD3+ cells was counted in 4-cross sections per valve. All analyses were performed double blindly without prior knowledge of the genotype.

Statistical analysis

All data are presented as geometric mean with 68% confidence interval (CI), which represents one standard deviation from the geometric mean if a log-normal distribution is assumed. Data are analysed by means of the Mann-Whitney *U* test (Graphpad Software version 4.02, San Diego, CA). Frequency data for lesion classification were compared by means of the χ^2 test. $P < 0.05$ was regarded as statistically significant.

Results

General Characteristics of mϕ Specific LRP Deficient mice on an apoE$^{-/-}$ and LDLR$^{-/-}$ Background

DNA isolated from peritoneal macrophages from LysMCre^{+} LRP$^{flox/flox}$ apoE$^{-/-}$ LDLR$^{-/-}$ (mϕ LRP-) mice and control LRP$^{flox/flox}$ apoE$^{-/-}$ LDLR$^{-/-}$ (mϕ LRP+) littermates was subjected to PCR analysis to detect the presence of LysMCre/loxP-mediated recombination of LRP (ΔLRP). A 400-bp ΔLRP PCR product of DNA from these isolated peritoneal macrophages was present in the mϕ LRP- mice (Fig. 1, lane 1 and 2), whereas no PCR product was detected in peritoneal macrophages of mϕ LRP+ mice (Fig. 1, lane 3 and 4), indicating successful recombination of LRP in macrophages in the mϕ LRP- mice only. All mice appeared healthy and displayed no signs of abnormalities. Mean body weight, plasma cholesterol and triglyceride levels, and plasma lipoprotein distribution were similar between mϕ LRP- mice and control mϕ LRP+ littermates (Table I and Fig. I, online available at http://atvb.ahajournals.org). The circulating CD45+ (total blood leukocytes), CD3+ (T cells) and CD19+ (B cells) cells were similar between the mϕ LRP- mice and control mϕ LRP+ littermates (Table 1, online available at http://atvb.ahajournals.org). Likewise, the CD11b+ monocyte and CD11b+ granulocyte populations were also similar between the mϕ LRP- mice and control mϕ LRP+ littermates (Table 1, online available at http://atvb.ahajournals.org).

Table I Composition of atherosclerotic lesions

	mϕ LRP+		mϕ LRP-	
	lesion content (x 10^3 μm^2)	% of total lesion area†	lesion content (x 10^3 μm^2)	% of total lesion area†
AIA-31240-positive area	76.0 (66.3-87.1)	65 (63-67)	146.0 (124.4-171.2)**	65 (62-67)
α-actine 1A4-positive area	9.8 (7.19-12.1)	24 (23-26)	17.6 (13.7-22.6)	27 (26-29)
Sirius red-positive area	14.8 (11.5-19.0)	20 (17-23)	27.2 (20.8-35.6)*	34 (30-39)**

Macrophage (AIA-31240-positive), SMC (α-actine-positive) and collagen (Sirius red-positive) were expressed as absolute area and as percentage of total lesion area. †Since SMC and collagen stainings may overlap, the sum of the AIA-31240- and α-active 1A4- Sirius red-positive area may exceed 100%. Data represent geometric mean and 68% CI. *P < 0.05, **P < 0.005, statistically significant from mϕ LRP+ littermates.

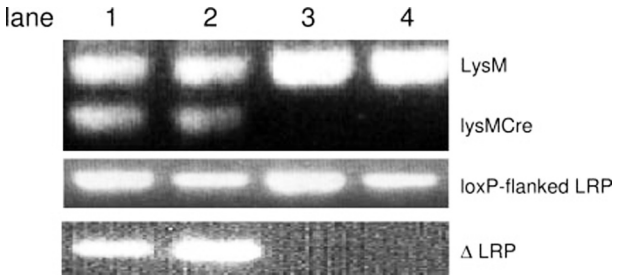

Figure 1 LysMCre/loxP-mediated recombination of LRP in thioglycollate-elicited peritoneal macrophages. Successful LysMCrelox/P-mediated recombination of LRP was detected in isolated peritoneal macrophages from mϕ LRP- mice (lane 1 and 2). No recombination was detected in peritoneal macrophages from mϕ LRP+ mice (lane 3 and 4). LysM: lysozymel M, LysMCre: lysozyme M Cre recombinase

Effect of mϕ Specific LRP Deficiency on Atherosclerotic Lesion Size

We investigated the role of LRP in macrophages in the development of atherosclerosis on an apoE and LDLR double deficient background. The total atherosclerotic lesion area was significantly increased in mϕ LRP- mice as compared to control mϕ LRP+ littermates at 18-week of age [geometric mean (68% CI): 24.2 (20.8-28.2) x10^4 μm^2 and 11.3 (9.6-13.3) x10^4 μm^2 for mϕ LRP- and mϕ LRP+, respectively, P < 0.001, Fig. 2A]. In addition, mϕ LRP- mice showed a significant increase in lesion severity as compared to control mϕ LRP+ littermates (Fig. 2B). Mϕ LRP- mice had a significant lower incidence of early lesions (4.0% vs. 18.7%, P < 0.05), an equal percentage of moderate lesions (18.0% vs. 18.7%) and a significant higher incidence of advanced lesions (78.0% vs. 62.7%, P < 0.05) as compared to the control mϕ LRP+ littermates. These data indicate that LRP deficiency in macrophages results in increased atherosclerotic lesions in mice.

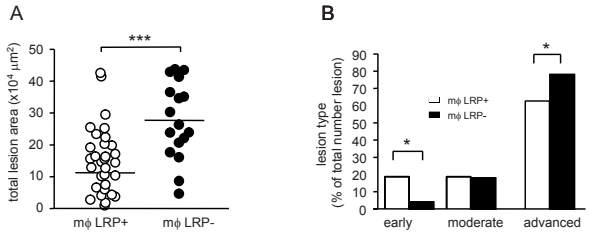

Figure 2 **Quantitative assessment of atherosclerosis at 18-week of age**
(A) Atherosclerosis lesion area of mϕ LRP- mice () and control mϕ LRP+ () littermates quantified at the level of the aortic root and (B) lesion categorisation of mϕ LRP- mice (black bars) and control mϕ LRP+ littermates (open bars) shown as percentage of the total number of lesions. Line represents geometric mean area for each group. Frequency data were compared by means of the $\chi 2$ test; *P<0.05, ***P<0.001, statistically significant from different control mϕ LRP+ littermates.

Effect of mϕ Specific LRP Deficiency on Atherosclerotic Lesion Composition

To investigate whether the LRP in the macrophages also affects the composition of the atherosclerotic lesions, we determined the percentage of macrophages (AIA-31240-positive area), SMC (α-actin-positive area) and collagen (Sirius red-positive area) content in the individual atherosclerotic lesions of 18-week old mϕ LRP- mice and control mϕ LRP+ littermates. As expected from the increased total lesion area in the mϕ LRP- mice, the total macrophage and collagen lesion contents were increased in the mϕ LRP- mice as compared to control mϕ LRP+ littermates (Table 1). The total SMC lesion content was also higher in the mϕ LRP- mice as compared to mϕ LRP+ littermates, although this was not statistically significant. To analyse the macrophage, SMC and collagen content independent of the total lesion area, we corrected the lesion content for the total lesion area at the level of individual lesions. The percentages of macrophage and SMC in the lesions were similar between the mϕLRP- and control mϕ LRP+ littermates (Table 1). However, the mean percentage of collagen in the lesions per mouse was significantly increased in mϕ LRP- mice as compared to control mϕ LRP+ littermates (Table 1, Fig. 3A). Furthermore, the percentage of collagen was also significantly increased in the individual advanced lesion of mϕ LRP- mice as compared to control mϕ LRP+ littermates, indicating that the increased collagen is also independent of lesion severity (Fig. 3B). This is clearly illustrated by the representative photomicrographs of atherosclerotic lesions of mϕ LRP+ and mϕ LRP- mice (Fig. 3C and 4D). Taken together, the relative macrophage and SMC content of mϕ LRP- mice do not differ, but the atherosclerotic lesions contain higher collagen content as compared to control mϕ LRP+ littermates.

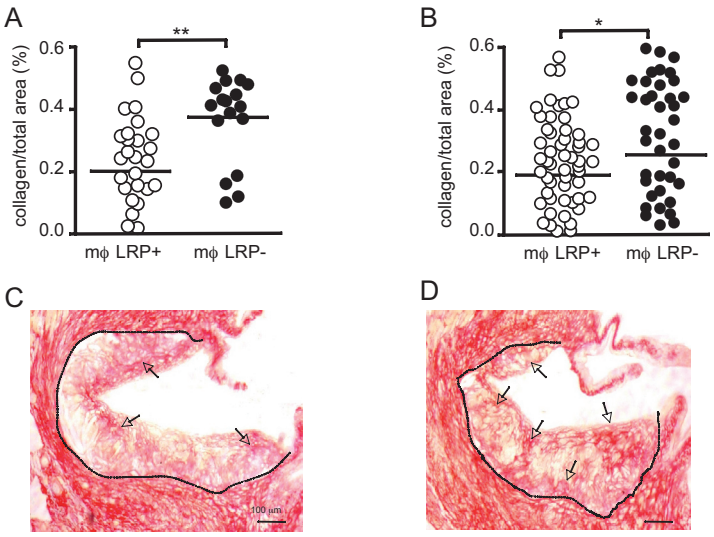

Figure 3 Collagen content of atherosclerotic lesions. (A) Percentage collagen (Sirius red-positive) area of mφ LRP- (●) and mφ LRP+ (○) littermates quantified at the level of the aortic root. Every dot represents the average of 3 lesions per mouse (B) Percentage collagen (Sirius red-positive) area in individual advanced lesions of mφ LRP- (●) and mφ LRP+ (○) littermates quantified at the level of the aortic root. Every dot represents 1 les Lines represent geometric mean area for each group. Representative photomicrographs of size-matched lesion of mφ LRP+ (C) and mφLRP- (D) stained with Sirius red. Dashed lines represent the border of the atherosclerotic lesions. Arrows indicate intensive accumulation of collagen area. *P<0.05 **P<0.01, statistically significant from control mφ LRP+ littermates.

Potential Mechanism Contributing to Increased Collagen Accumulation in Atherosclerotic Lesions of mϕ Specific LRP deficient mice

Collagen content in the extracellular matrix can be controlled amongst others by the matrix metalloproteinase (MMP)/tissue inhibitor metalloproteinase (TIMP) system and interferon-γ (INF-γ).[28,29] MMP-9 is an important representative of the MMP/TIMP system that may be involved in collagen degradation in the extracellular matrix of atherosclerotic lesions. On the other hand T cells can produce IFN-γ, a modulator of collagen synthesis. Therefore, we explored the MMP-9 content and T cells in the atherosclerotic lesions. The total MMP-9 area was significantly increased in the mϕ LRP- mice as compared to control LRP+ littermates [geometric mean (68% CI): $18.5x10^3$ ($14.5-23.8x10^3$) and $11.6x10^3$ ($10.0-13.6x10^3$) μm^2 for mϕ LRP- and mϕ LRP+ mice, respectively, $P < 0.05$]. When corrected for the total lesion area at the level of individual lesions, the percentage of MMP-9 content was similar in mϕ LRP- and mϕ LRP+ mice [geometric mean (68% CI): 0.21 (0.17-0.25) and 0.29 (0.26-0.32) % for mϕ LRP- and mϕ LRP+ mice, respectively, P =0.12]. The total number of CD3+ T cells in the lesions of the mϕ LRP- and mϕ LRP+ littermates was similar (Fig. 4A). However, the number of CD3+ T cells corrected for lesion size was significantly decreased in the mϕ LRP- mice as compared to the mϕ LRP+ littermates (Fig. 4B). Thus, relative MMP-9 content does not differ between mϕ LRP- mice and mϕ LRP+ littermates, whereas the relative number of CD3+ T cells is significantly decreased in the atherosclerotic lesions of mϕ LRP- mice.

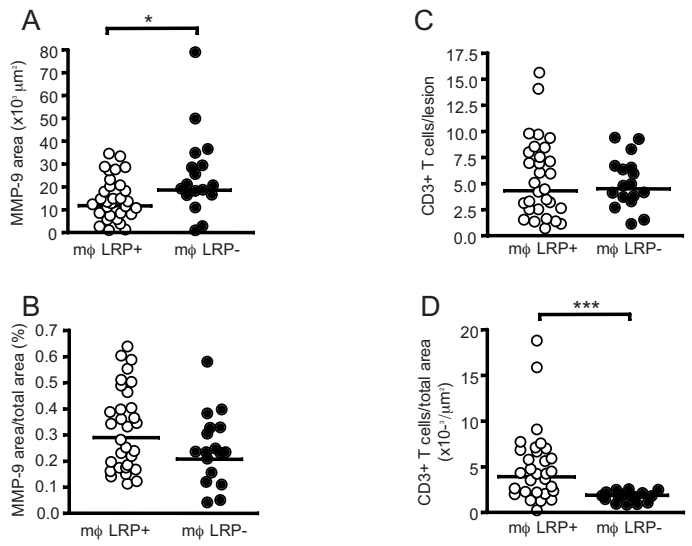

Figure 4 D3+ T cell content of atherosclerotic lesions. (A) Total number of CD3+ T cells and (B) percentage of CD3+ T cells in lesions of mφ LRP- (●) and mφLRP+ (○) littermates quantified at the level of the aortic root. Every dot represents the average of 3 lesions per mouse. ***P<0.001, statistically significant from control mφLRP+ littermates.

Discussion

LRP has been shown to have a distinct atheroprotective role at the level of the liver and SMC.[7,12] However, to date, the role of LRP in the macrophages, the key mediators in the pathogenesis of atherosclerosis has not been elucidated *in vivo*. In the present study, we investigated the role of LRP in macrophages in the development of atherosclerosis *in vivo* independent of its classical role in the uptake of lipoproteins via the apoE- and LDLR-mediated pathway. We demonstrated that macrophage specific LRP deficiency leads to a significant increase in atherosclerosis. In addition, macrophage specific LRP deficient mice exhibit an increase in relative collagen content, whereas the macrophage and SMC contents of the plaques were not affected. Furthermore, the plaques of the macrophage specific LRP deficient mice contain less relative numbers of CD3+ T cells, whereas the MMP-9 content was not different. We conclude that, like LRP from the liver and SMCs, macrophage LRP has an atheroprotective potential in apoE LDLR double knockout mice.

Our results are in apparent contradiction with previous *in vitro* studies, showing that macrophage LRP has pro-atherogenic properties. LRP has a well-established role in the apoE-mediated uptake of remnant lipoproteins and has thereby pro-atherogenenic potentials in the macrophages.[4,6,30] Furthermore, macrophage LRP is demonstrated to mediate the adhesion and migration of leukocytes,[15] formation of atherogenic oxidised LDL,[16,17] and the clearance of

pro-atherogenic ligands, such as tissue-type plasminogen activator,[12,31] and plasminogen activator inhibitor-1.[32] All these processes promote the formation of foam cell in vitro, which point to less atherosclerosis in the absence of macrophage LRP. However, we observed increased atherosclerosis macrophage specific LRP deficient mice, despite the exclusion of possible apoE- and LDLR-mediated uptake of pro-atherogenic remnant lipoproteins (Table 1, Fig. 2). The difference between the previous studies[15-17] and our current study may be due to the differences in the study designs. *In vitro* isolated macrophage systems were used in the previous studies, whereas an *in vivo* mouse model was used in the present study. In our *in vivo* model LRP exhibits both pro-atherogenic and anti-atherogenic properties. Our results demonstrate that the anti-atherogenic properties of LRP in the macrophages dominate the pro-atherogenic properties in apoE and LDLR double deficient mice.

The mechanism by which macrophage LRP modulates atherosclerosis is not clear. Firstly, since LRP recognises >50 distinct pro-atherogenic and anti-atherogenic ligands, it can be postulated that the increased atherosclerosis in macrophage LRP deficient mice might be due to accumulation of pro-atherogenic LRP ligands locally in the vascular wall or in the plasma. Pro-atherogenic LRP ligands include coagulation factor VIII,[33] von Willebrand factor [34] and tissue type plasminogen activator. [31,35] We have previously shown that disruption of the hepatic LRP gene results in increased plasma levels of these LRP ligands. Secondly, recent work has implicated LRP in several signal transduction pathways including the regulation of cell migration and the remodelling of the extracellular matrix. In the present study, we show that deletion of the LRP gene in macrophages leads to increased collagen content of atherosclerotic lesion independent total area, whereas no differences were observed in SMC content (Fig. 3, Table 1). The collagen content in the extracellular matrix can be controlled by the MMP/TIMP system and activated T cells.[28,29] Whereas we observed no differences in MMP-9 content in the lesions, the relative number of T cells is significantly lower in the atherosclerotic lesions of the macrophage specific LRP deficient mice (Fig. 4). Possibly, the regulation of lesional collagen as modulated by T cells is impaired in the absence of LRP in the macrophages. Obviously, further detailed studies are required to gain additional insight into the underlying mechanisms how LRP affects T cell number and collagen content, and their possible interaction.

Our data provide evidence that LRP has an atheroprotective potential in the apoE LDLR double knockout mice at the level of macrophages in addition to the previously shown anti-atherogenic characteristics of LRP in the liver and SMC, and may modulate the extracellular matrix in the atherosclerotic lesions.

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Acknowledgments

This work was supported by The Netherlands Heart Foundation NHS 2000.051 (L.S.M.B.), NHS 2005 B226 (J.F.P.B.), The Netherlands Organization for Scientific Research NWO 902-26-242 (B.J.M.v.V.), The Emmy Noether Program of the Deutsche Forschungsgemeinschaft (P.M.), NIH (J.H.) and the Wolfgang Paul Program of the Humboldt Foundation (J.H.). B.J.M. van Vlijmen is a fellow of the Royal Netherlands Academy of Arts and Sciences.
We like to thank Anita van Nieuwkoop and A. Susanne M. Zadelaar for their excellent technical support.
All authors have no conflict of interest.

ONLINE SUPPLEMENT

Macrophage Low-density Lipoprotein Receptor-related Protein Deficiency Enhances Atherosclerosis in ApoE/LDLR Double Knockout Mice

Table **Body weight, plasma lipid levels and blood leukocytes of 18-weeks old of mϕ LRP- mice and mϕ LRP+ littermates.**

	mϕ LRP+	mϕ LRP-
Body weight (g)	21.1 (20.7-21.4)	20.9 (20.5-21.3)
Plasma cholesterol (mM)	23.2 (22.3-24.2)	21.9 (21.0-22.9)
Plasma triglyceride (mM)#	1.8 (1.7-1.9)	1.6 (1.5-1.8)
CD45+ (106 cells/mL)	13.4 (12.5-14.4)	13.3 (11.9-14.9)
CD19+ (106 cells/mL)	6.8 (6.3-7.4)	6.5 (5.9-7.3)
CD3+ (106 cells/mL)	4.6 (4.1-5.0)	4.1 (3.6-4.7)
CD11b+ monocytes (106 cells/mL)	1.0 (0.9-1.0)	1.0 (0.9-1.1)
CD11b+ granulocytes (106 cells/mL)	1.7 (1.6-1.9)	1.6 (1.5-1.7)

Data represent geometric mean and 68% CI. #Sum of free and triglyceride-derived glycerol.

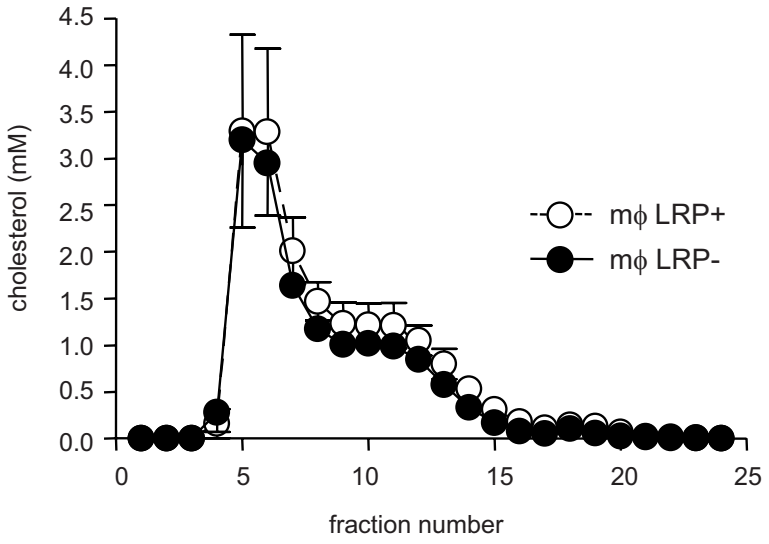

Figure **Plasma lipoprotein distribution.**
Plasma lipoprotein profiles of mϕ LRP- mice () and control mϕ LRP+ () littermates were size-fractionated by FPLC followed by the determination of the individual fractions. Identical lipoprotein distribution is observed between mϕ LRP- mice and control mϕ LRP+ littermates. VLDL: very-low density lipoprotein, LDL: low density lipoprotein, HDL: high density lipoprotein.

Chapter 6

Decreased circulating endothelial progenitor cell counts accompany elevated CRP levels in subjects with the metabolic syndrome without overt cardiovascular disease

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Submitted for publication

Abstract

To assess the influence of inflammation on circulating endothelial progenitor cells (EPC) and haematopoietic stem cells (HSC) in relation to carotid atherosclerosis in non-diabetic patients with metabolic syndrome (MS) without manifest cardiovascular disease (CVD). Seventy three male non-diabetic patients with MS without manifest CVD were included. A threshold of 1.8mg/L was used to define elevated (CRP+) and low (CRP-) CRP levels. Number of HSCs and EPC were quantified by fluorescence-activated cell sorter analysis. Cytokine and adhesion molecule profiles were assessed. Carotid atherosclerosis and adipose tissue distribution was assessed by MRI. Significantly lower number of EPC (40%, p = 0.001) and HSC (61%, p = 0.006) were found in CRP+. Patients with atherosclerotic plaques and CRP+ had lower HSC (p = 0.005) and EPC (p = 0.07) compared to CRP-. Subcutaneous adipose tissue area was higher in CRP+ at the waist and the hip (25%; p = 0.002 and 21%; p = 0.005 respectively). In a multiple linear regression analysis TNF-α (ß:-0.02; SE: 0.009; p = 0.03) and waist circumference (ß:-0.016; SE: 0.008; p = 0.049) were found as explanatory variables for EPC counts; P-selectin (ß:-0.007; SE: 0.002: p < 0.001) and TNF-α (ß: -0.024; SE: 0.008; p = 0.025) for HSCs.

In conclusion, decreased CEPC and HSC numbers were shown to accompany elevated CRP levels in non-diabetic patients with MS without manifest cardiovascular disease. This relation was observed in subjects with atherosclerotic plaques and elevated CRP levels but not in the absence of plaques.

Introduction

Circulating endothelial progenitor cells (EPC) are vasculogenic cell populations present in the mononuclear fraction of peripheral blood, which are thought to be derived from hemangioblastic cell that reside in the bone marrow. EPC can incorporate into the endothelial monolayer, stimulate proliferation of neighbouring endothelial cells and induce the formation of new blood vessels.[1,2] Significantly lower numbers of EPC have been shown in subjects with a high risk for cardiovascular disease[3] and in subjects with overt cardiovascular disease[4], especially in combination with the metabolic syndrome.[5] In addition, EPC may have prognostic[6] and therapeutic potential.[7,8] The relation between EPC and the extent of atherosclerotic disease burden is not yet clearly established.

The metabolic syndrome (MS) is associated with increased cardiovascular morbidity and mortality[9,10]. MS and visceral obesity are also associated with increased plasma C-reactive protein (CRP) levels[11-13]. CRP is a marker of the systemic inflammatory state and is shown to be associated with endothelial dysfunction[14], atherosclerosis[15,16], and future cardiovascular events[17]. CRP attenuates EPC survival, differentiation and function *in vitro*[18]. Furthermore, CRP levels have been related to circulating cytokine levels such as TNFα and IL6 in patients with characteristics of the metabolic syndrome. These key inflammatory cytokines have previously been linked to endothelial function and the regulation of bone marrow derived vasculogenic cells[19-21]. The influence of systemic inflammation on these cellular markers of endothelial dysfunction has not been studied *in vivo* in patients with the metabolic syndrome. We hypothesized that patients with MS and elevated systemic inflammation would have decreased EPC counts compared to MS patients with low systemic inflammation. Therefore, we assessed the influence of systemic inflammation on EPC and HSC counts in relation to the extent of carotid atherosclerosis in subjects with the metabolic syndrome without diabetes or overt cardiovascular disease. In addition, we explored prevailing cytokine and adhesion molecule levels. MRI assessments of the carotid artery and adipose tissue distribution were used to obtain measures of obesity and atherosclerotic disease burden.

Methods

Study design & Subjects

We included 73 male subjects above 50 years of age with visceral obesity and the metabolic syndrome according to the International Diabetes Federation criteria.[22] Exclusion criteria were type 2 diabetes mellitus, manifest cardiovascular disease, use of statins or non-steroidal anti-inflammatory drugs, current smoking, familial history of premature cardiovascular disease, severe obesity(BMI>40 kg/m^2), and contraindications for MRI. The subjects were divided into two groups with varying inflammatory states defined by the previously

published mean of CRP in male subjects as threshold (i.e. 1.8 mg/L)[23]. The study complies with the Declaration of Helsinki and was approved by the institutional review committee and all subjects gave informed consent. HdB was supported by the Dutch Heart Foundation (Grant NHS2006B106)

Anthropometric and laboratory assessments

Screening of potential candidates was performed by medical history, anthropometry and selected laboratory values. Extensive laboratory assessments were performed in all included subjects within two weeks of MRI assessments. These values were used in the statistical analysis. Blood pressure was assessed using an automatic blood pressure monitor (Omron 705IT, Hoofddorp, The Netherlands). Three measurements were averaged for use in the analysis. Blood samples were collected after a 12 hour overnight fast for chemical and haematological laboratory assessments. The high sensitive CRP assay was performed with the Tina Quant C-reactive protein (latex) high sensitive assay (Roche, Basel, Switzerland). Participants with CRP levels above 15mg/L were regarded as having an intercurrent infection and were not included in the assessments. Insulin was determined in heparinised plasma using a solid-phase, two-site chemiluminescent immunometric assay carried out on an Immulite 2500(DPC, Los Angeles, USA).

Cytokines and adhesion molecules

The serum levels of cytokines and adhesion molecules were measured using a Randox Evidence Investigator and the Cytokine & Growth Factors Biochip Array and Adhesion Molecules Biochip Array. The cytokine array contains discrete test regions of immobilized antibodies specific to IL-1α, IL-1β, Il-2, IL-4, IL-6, IL-8, IL-10, IFN-γ, TNF-α. The adhesion molecule array contains antibodies specific to soluble E-, L- and P-selectin, ICAM-1 and VCAM-1. The light signal generated from each test region on the Biochip with antibodies labelled with Horse Radish Peroxidase is detected using a super cooled charge coupled device camera and compared to that from a stored calibration curve. Sample preparation in short: the samples were diluted with assay buffer or diluent and applied to a biochip (well). The biochip (carrier) was incubated at 37 °C and shaken at 370 rpm at a thermoshaker for 60 min. After washing the conjugate (HRM labelled antibodies) was added and again incubated at 37°C and shaken at 370 rpm at a thermoshaker for 60 min. After washing 250 µl of a 1:1 mix of luminol and peroxide was added and incubated for 2 minutes. Finally the carrier is imaged using an Investigator System conform the manufacturers instruction.

3T Magnetic Resonance Imaging Protocol

Carotid Imaging: Magnetic resonance imaging was performed on a 3T scanner (Philips, Achieva, Best, The Netherlands) as previously described and validated[24]. In short, after performing the preparatory scan sequences, a dual inversion recovery (black-blood), spoiled segmented k-space fast gradient echo sequence with spectral selective fat suppression

was used to obtain ten contiguous transverse slices covering 2 cm of the carotid bulb and the common carotid artery. In all subjects, images were acquired from the left carotid artery. Images were analysed using the Vessel MASS software package developed at our institution, as described previously[25]. Atherosclerotic disease burden was assessed by the total vessel wall area of the scanned vessel segment (Total VWA). All scans were also visually scored for the presence of atherosclerotic plaques as follows: 0: no plaques, 1: focal asymmetrical vessel wall thickening < 2x the opposite vessel thickness, 2: focal asymmetrical vessel wall thickening ≥ 2x & < 3x the opposite vessel thickness. 3: focal asymmetrical vessel wall thickening ≥ 3x the opposite vessel thickness. When more than one plaque was present, the largest was used for classification.

Adipose tissue imaging: Subjects were positioned in the magnet in a supine position. The body coil was used for obtaining the images. A sagittal single shot gradient echo sequence survey scan was used for the imaging of the vertebral column in the lumbar region. Subsequently, a second single shot gradient echo sequence in the transversal plane was used for obtaining three contiguous slices of 10mm without angulations. The slices were centred at the intervertebral disk level between the 4^{th} and 5^{th} lumbar vertebra. The following parameters were used for image acquisition: echo time 3.7 ms (TE), repetition time7.5 (TR), pulse angle 45 degrees, and 2 signal averages were performed. The images were obtained with 3 breath holds of 6 seconds. The field of view was 500mm. A voxel size of 1mm x 1.3mm x 10mm was obtained. Images were assessed using the MASS software package developed at our institution allowing a semi-automated detection of subcutaneous (SAT) and visceral adipose tissue (VAT) area.

Enumeration of EPC and HSCs

Enumeration of circulating hematopoietic stem cells (HSCs) and circulating endothelial progenitor cells (EPC) was performed as recently described.[26] This method uses Trucount tubes that contain a defined number of brightly fluorescent microbeads, permitting the acquisition of absolute counts of cells, even at very low numbers. Circulating HSCs are defined as cells with low-expression of CD45, positive for CD34, and located in the lympho-gate on a side- and forward-scatter plot. This gating strategy was extended by calculating the number of $CD34^+$ cells that also express vascular endothelial growth factor receptor-2 (VEGFR-2) to define the number of EPC. This strategy avoids inclusion of mature endothelial cells, which are also positive for CD34 and VEGFR-2, since they are located outside the lympho-gate.

Statistical analysis

Continuous variables are presented as mean values ± standard error or as medians and interquartile ranges if the assumption of normality was not met. Comparisons between continuous variables were performed with independent samples *t* tests or Mann-Whitney *U* tests when not normally distributed. Correlations were analyzed with bivariate correlation analysis (Pear-

son's or Spearman's correlation depending on distribution). Logarithmically transformed HSCs and EPC were used to compare the influence of CRP levels in groups with and without atherosclerotic plaques and in multiple linear regression analyses. Multiple linear regression analysis was performed to evaluate the determinants of precursor cell counts.. Variables were selected from all determined anthropometric, laboratory, cytokine profiles, and MRI determined adipose tissue distribution if the obtained p-value in a univariate linear regression analysis was lower than 0.1. The selected variables were used in a multiple regression analysis with forward selection. Analyses were performed using SPSS version 12.01 (SPSS, Chicago, Illinois, USA). All analyses were two-sided, with a level of significance of α=0.05.

Results

Patient characteristics

Of the 73 included participants in the study, 51 subjects had elevated CRP levels (CRP+), and 22 subjects had low CRP levels (CRP-). Patient characteristics are shown in **table 1**. There were no differences between both groups in age, systolic, and diastolic blood pressures, and total cholesterol levels. Subjects with elevated CRP levels had significantly higher waist circumferences (p = 0.01), body weight (p = 0.005), HDL cholesterol levels (p = 0.028), and LDL cholesterol levels (p = 0.02) in comparison to subjects with low CRP levels.

Table 1 Patient characteristics

	CRP- n = 22	CRP+ n = 51	*p*
Age (years)*	58.9 (1.14)	59.2 (0.68)	ns
Waist circumference (cm)	103.5 (99.8-106.9)	108 (102-116)	*0.01*
Body weight (kg)	87.6 (81.9-94.2)	97.1 (87.9-107.3)	*0.005*
Systolic BP (mmHg)*	151.8 (4.0)	151.1 (2.5)	ns
Diastolic BP (mmHg)*	88.5 (1.9)	89.1 (1.1)	ns
TC (mmol/L)*	5.6 (0.2)	5.9 (0.13)	ns
HDL (mmol/L)	1.11 (1.02-1.29)	1.31 (1.11-1.54)	*0.028*
TG (mmol/L)	2.30 (1.49-3.49)	1.68 (1.24-2.13)	ns
LDL (mmol/L)	3.32 (2.64-3.75)	3.70 (3.22-4.30)	*0.02*
FBG (mmol/L)	5.2 (4.7-5.4)	5.1 (4.9-5.7)	ns
HbA1c*	4.9 (0.1)	5.1 (0.1)	ns
Insulin (mU/L)	8.5 (6.0-12.4)	10.0 (7.0-15.0)	ns
HOMA	1.1 (0.7-1.7)	1.4 (0.9-2.0)	ns
CRP (mg/L)	0.94 (0.56-1.47)	2.46 (1.59-3.29)	*0.000*
Framingham*	14.15 (1.34)	13.02 (0.60)	ns

CRP- and CRP+ data are presented as median (interquatile range).*Data are presented as mean (SE). BP: blood pressure, TC: total cholesterol, HDL: high-density lipoprotein, TG: triglycride, LDL low density lipoprotein, FBG: fasting blood glucose, HOMA: HOMA insulin resistance index, CRP: C-reactive protein

EPC and HSCs (Table 2)

EPC and HSCs were significantly decreased in CRP+ compared to CRP- (**Figure 1**). Furthermore, the EPC/HSC ratio was observed to be significantly lower in CRP+ in comparison to CRP- ($p = 0.022$).

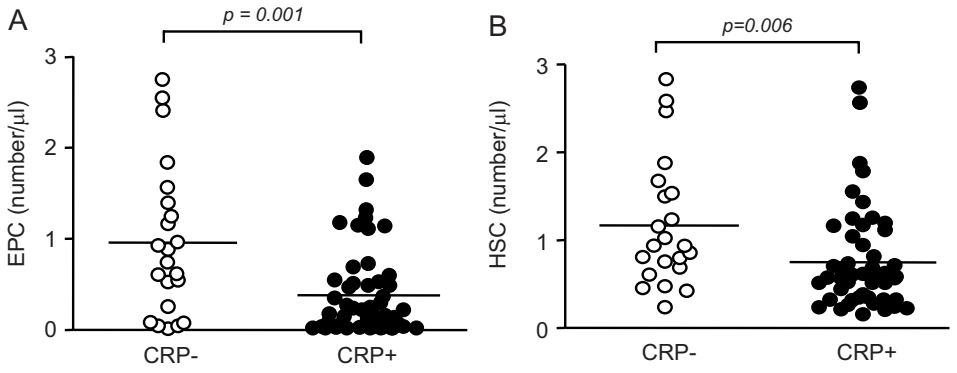

Figure **EPC (A) and HSC (B) numbers in patients with elevated CRP (CRP ≥ 1.8 mg/L, CRP+) and low LRP (< 1.8 mg/L, CRP-) levels.**

Table 2 **Circulating endothelial progenitor cell and haematopoietic stem cell numbers.**

	CRP- (n = 22)	CRP+ (n = 51)	*p*
EPCs (number/µl)	0.81 (0.21-1.43)	0.18 (0.02-0.51)	0.001
HSCs (number/µl)	0.93 (0.66-1.57)	0.59 (0.34-1.04)	0.006
CEPC/HSC ratio	0.90 (0.39-0.95)	0.47 (0.03-0.92)	0.022

Data are presented as median (interquartile range). EPC: circulating endothelial progenitor, HSC: haematopoietic stem cells

Cytokine and adhesion molecule profiles (Table 3)

Plasma concentrations of IL-6 and TNFα were significantly higher in CRP+ vs CRP-, 2.4 pg/ml [1.90-5.40] vs 1.20 pg/ml [0.88-2.28] and 7.80 pg/ml [0-10.30] vs 6.40 pg/ml [0-7.73] respectively. Plasma levels of other cytokines and adhesion molecules did not differ significantly between the two groups. Significant correlations were observed between EPC and IL-6 (r:-0.32, $p = 0.007$) and TNF-α (r:-0.26, $p = 0.029$). HSCs correlated significantly with CRP (r:-0.27, $p = 0.02$), IL-6 (r:-0.27, $p = 0.02$), TNFα (r:-0.26, $p = 0.026$), ICAM-1 (r:-0.33, $p = 0.004$) and P-selectin (r:0.39, $p = 0.001$).

Table 3 The cytokine and adhesion molecule profiles

	CRP- (n = 20)	CRP+ (n = 51)	*p*
IL-1α (pg/ml)	0.50 (0-0.85)	0 (0-0.80)	ns
IL-1β (pg/ml)	0.85 (0-1.73)	0 (0-1.8)	ns
IL-2 (pg/ml)	0 (0-6.05)	0 (0-9.70)	ns
IL-4 (pg/ml)	4.80 (3.18-5.10)	5.2 (4.80-6.50)	ns
IL-6 (pg/ml)	1.20 (0.88-2.28)	2.4 (1.90-5.40)	*0.01*
IL-8 (pg/ml)	9.40 (6.28-11.53)	9.5 (7.20-11.80)	ns
IL-10 (pg/ml)	0 (0-0)	0 (0-0)	ns
INF-γ (pg/ml)	0 (0-6.18)	0 (0-6.70)	ns
TNF-α (pg/ml)	6.40 (0-7.73)	7.80 (0-10.30)	*0.024*
VCAM-1 (ng/ml)	509.80 (468.15-630.95)	490.40 (415.80-564.90)	ns
ICAM-1 (ng/ml)	305.70 (258.48-372.55)	303.10 (274.60-359.00)	ns
E-Selectin (ng/ml)	16.50 (12.88-20.55)	16.60 (12.50-20.90)	ns
P-Selectin (ng/ml)*	154.01 (8.54)	152.74 (5.66)	ns
L-Selectin (ng/ml)	988.25 (892.15-1110.28)	994.50 (901.10-1097.50)	ns

Data are presented as median (interquartile range). *Data are presented as mean (SE)

Table 4 MRI assessed atherosclerotic and adipose tissue distribution

	CRP- (n = 22)	CRP+ (n = 51)	*p*
Total VWA (cm²)	4.40 (3.85-5.46)	4.53 (4.07-5.30)	ns
Plaque 0 no. (%)	7 (31.8)	8 (15.7)	ns
Plaque 1 no. (%)	8 (36.4)	25 (49.0)	ns
Plaque 2 no. (%)	5 (22.7)	12 (23.5)	ns
Plaque 3 no. (%)	2 (9.1)	6 (11.8)	ns
VAT (cm²)	388.0 (300.6-515.4)	448.9 (348.8-563.8)	ns
Waist SAT (cm²)	649.4 (512.3-722.1)	761.1 (671.8-983.8)	*0.001*
Hip SAT (cm²)	475.8 (391.5-574.4)	582.1 (510.1-707.9)	*0.002*

Data are presented as median (interquartile range). VWA: vessel wall area, VAT: visceral adipose tissue, SAT: subcutaneous adipose tissue

3T MRI assessments (Table 4)

The extent of carotid atherosclerotic disease burden did not significantly differ between the two groups. No difference was observed in the number of atherosclerotic plaques between CRP+ and CRP-. Subjects with atherosclerotic plaques and elevated CRP levels had statistically significant lower HSCs [0.59/μl (1.87) vs 1.07/μl (1.91), $p = 0.005$] and non-significantly lower EPC [0.22/μl (4.69) vs 0.55/μl (5.24), $p = 0.07$] compared to in CRP-. No difference was seen in HSC and EPC counts in subjects without atherosclerotic plaques. Increased deposits of subcutaneous adipose tissue were observed in CRP+ both at the waist ($p = 0.001$) and the hip ($p = 0.002$). Visceral adipose tissue did not vary between the two groups.

Multiple regression analysis

Multiple linear regression analysis was performed to adjust for possible confounders influencing the observed differences in EPC and HSC counts as described in *Methods*. For EPC, waist circumference, CRP, hip SAT, and TNFα, had p-values <0.1 in the univariate linear regression analyses and were used as independent variables in the multiple linear regression analysis with forward selection. The obtained model consisted of TNFα (ß:-0.02; SE:0.009; $p = 0.03$) and waist circumference (ß:-0.016; SE:0.008; $p = 0.049$) as explanatory variables. For the multiple linear regression analysis regarding HSC counts, the following parameters were used as independent variables: CRP, TNFα, ICAM, and P-Selectin. The final model obtained consisted of P-selectin (ß:-0.007; SE:0.002; $p < 0.001$) and TNFα (ß:-0.024; SE:0.008; $p = 0.025$) as explanatory variables.

Discussion

This study demonstrates that elevated CRP levels are accompanied by decreased counts of EPC, HSCs and EPC/HSC ratio in subjects with the metabolic syndrome without diabetes and without overt cardiovascular disease. Subjects with atherosclerotic plaque on MRI have lower HCS and EPC counts when low grade inflammation is present while this effect was not observed in the absence of plaques. Further exploration related the low cell counts to prevailing TNFα and p-Selectin concentrations.

Significantly lower EPC counts have previously been demonstrated in subjects with insulin resistance[27], and in subjects with high risk for cardiovascular disease[28], especially in combination with the metabolic syndrome[29]. We now studied subjects with the metabolic syndrome without DM2 or overt cardiovascular disease to test the hypothesis that systemic inflammation is the driver of low cell counts in these patients. Low grade systemic inflammation was indeed accompanied by significantly lower EPC and HSC counts in metabolic syndrome. This is in line with studies reporting low EPC in other chronic inflammatory disease states such as rheumatoid arthritis[30]. The populations compared in our study were significantly different in body weight, HDL-c, LDL-c, and CRP levels. To adjust for the possible effects of adipose tissue, we repeated the analysis after matching CRP- for body weight to a subset of CRP+. Metabolic, anthropometric profile and adipose tissue distribution were not different between CRP+ versus the CRP-. Also in this sub-analysis we observed significantly lower EPC and HSC counts CRP+ (Data not shown).

EPC are thought to be related to the atherosclerotic process.[31] Patients with proven coronary artery atherosclerosis have low EPC counts.[32] Similarly, low EPC counts have been reported in subjects with an increased risk of accelerated progression of atherosclerosis.[33] Furthermore, decreased EPC counts have been described in other conditions known to be

accompanied by an increased burden of atherosclerosis such as type 2 diabetes mellitus[34] and chronic kidney disease[35]. The exact relation between EPC and HSCs and atherosclerosis is not clearly established. We used carotid artery MRI to assess atherosclerotic burden, as previously described.[36] No statistically significant difference in atherosclerotic disease burden was observed between CRP+ and CRP-. The influence of CRP on HSC and EPC counts was also observed in patients with atherosclerotic plaques and not in the absence of plaques. We propose that EPC contribute to the maintenance of the structural stability of atherosclerotic lesions. Consequently, loss of EPC would result in increased plaque vulnerability. Lower EPC counts have been shown to be associated with higher future cardiovascular events.[37] Our study extends these finding by adding the effect of systemic inflammation on precursor cell counts. Thus inflammation, precursor cells, plaque vulnerability and events could be regarded as mutually dependent, causally linked phenomena.

We explored the intermediate metabolic and inflammatory pathways related to EPC and HSCs. TNFα and IL-6 levels were significantly different between the groups with varying CRP levels. TNFα and IL-6 also correlated with EPC and HSC counts. In multiple linear regression analysis, TNFα and waist circumference significantly determined EPC counts, whereas HSC counts were associated with serum P-selectin and TNFα concentrations. The role of IL-6 in the multiple linear regression analysis may have been underestimated in our study. The study population consisted of viscerally obese subjects only and visceral obesity (waist circumference) was shown to play an important role in determining EPC numbers. On the other hand, IL-6 is one of the cytokines most strongly related to visceral adiposity. This may have confounded the relation between IL6 and EPC counts. The pronounced effect of visceral obesity (waist circumference) as explanatory variable for EPC suggests other adipocytokines may affect precursor cell status in addition to IL-6 in these patients. TNFα and IL-6 have previously been linked to endothelial function [38,39]. TNFα has also been shown to inhibit EPC proliferation and differentiation *in vitro* in a dose dependent manner.[40] P-selectin contributes to the adhesive surface of activated platelets adhered to inflamed endothelial cells or extracellular matrix.[41,42] This surface is regarded to serve as the anchor place for circulating cells. Our *in vivo* observations relating cytokines and adhesion molecules to EPC and HSC counts are inline with these previous *in vitro* studies.

Our study has limitations. It was designed as a cross-sectional observational study only exploring circulating inflammatory cytokines and adhesion molecules. In addition, no in depth differentiation analysis of other bone marrow derived circulating cells was performed. However, our observations point to the relevance of EPC and HSCs in this patient category, and show for the first time the significance of low grade inflammation on fate of bone marrow derived cells in these patients. Future research may focus on the exact differentiation routes *in vivo,* including the effect of therapeutic manipulation of the systemic inflammation by for instance salicylates or thiazolidinediones.

In conclusion, lower counts of EPC and HSCs were shown to accompany elevated CRP levels in subjects with the metabolic syndrome without diabetes or overt cardiovascular disease. Interestingly, this was also observed in subjects with atherosclerotic plaques and not in the absence of plaques. If EPC are important for the vascular response to injury, systemic inflammation may profoundly affect this process.

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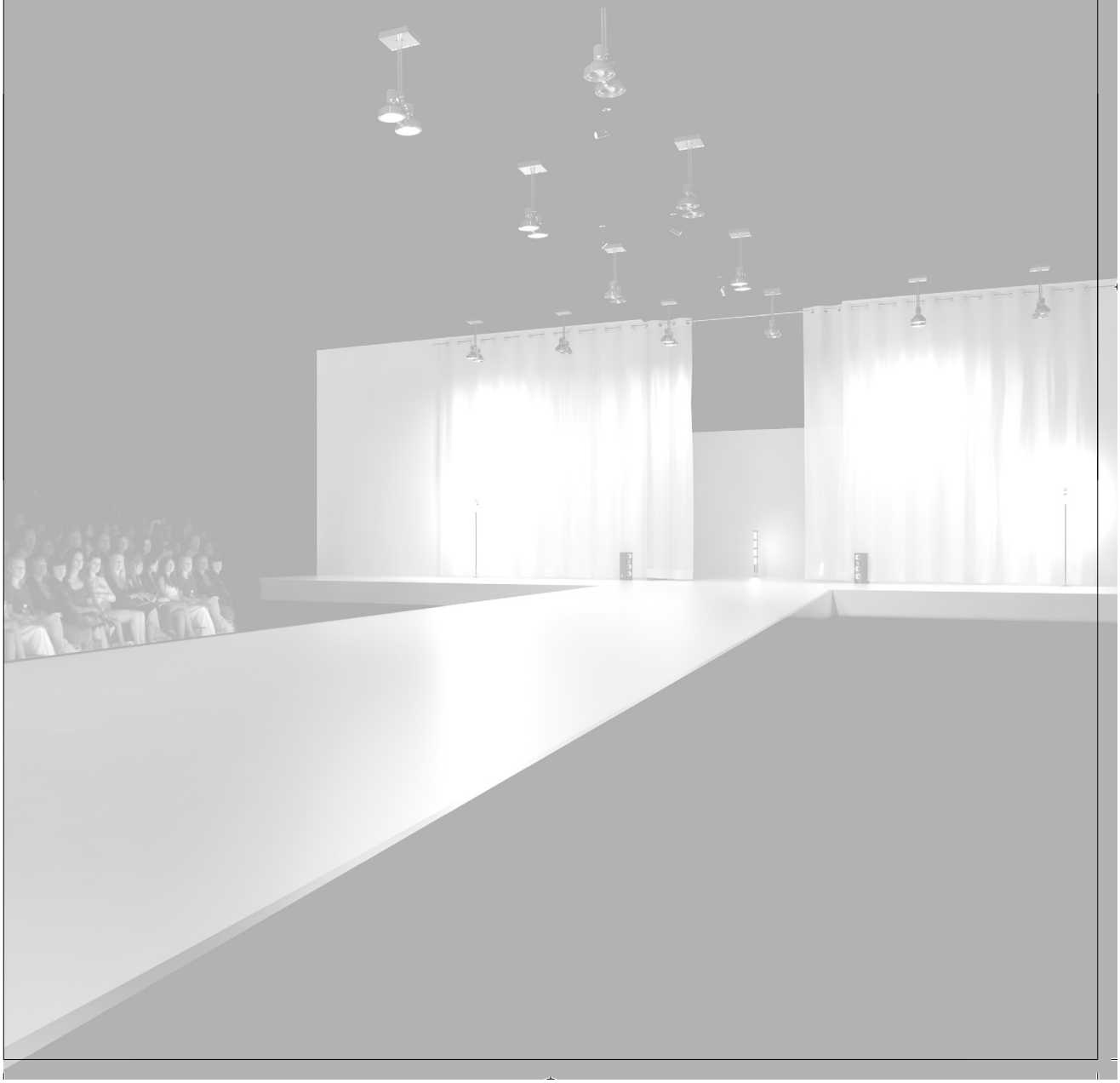
Chapter 7

General discussion



Overweight and obesity is reaching pandemic proportions and rapidly becoming a major health problem in Western societies, momentarily affecting more than 1 billion adults.[1]The pandemic of overweight and obesity is associated with an increased incidence of the metabolic syndrome (MetS). The MetS is now known to be associated with diabetes and increased cardiovascular morbidity and mortality.

The research described in this thesis was designed to gain further inside in the different aspects of the MetS. The studies were in particular directed to the physiological impact of plasminogen activator inhibitor-1 (PAI-1) and the low-density lipoprotein receptor-related protein (LRP) with respect to the MetS and atherosclerosis in mouse models. In addition, the effect of low-grade inflammation on the endothelial function in human subjects with MetS was addressed. The major conclusions and implications of our findings as well as the future perspectives will be discussed in this chapter.

PAI-1 catabolism and its role in insulin resistance and obesity

The low-density lipoprotein receptor (LDLR)-related protein (LRP) is a large cell-surface multi-ligand endocytic clearance and signalling receptor of the LDLR gene family.[2,3] LRP was originally identified as an endocytic receptor for apolipoprotein (apoE)-rich lipoproteins. LRP is known to recognise >50 structurally and functionally different ligands, including plasminogen activator inhibitor-1 (PAI-1).[4,5] PAI-1 is the primary inhibitor of the plasminogen activation *in vivo*. It is not known wether this PAI-1/LRP interaction is of physiological importance. Furthermore, it is unknown by what molecular mechanism PAI-1 is cleared from the circulation. By using the hepatic LRP deficient mouse model, we studied the role of LRP in the clearance of plasma PAI-1 (**Chapter 3**). We showed that plasma PAI-1 level is not regulated by LRP. However, inhibition of the LDLR family by receptor-associated protein (RAP) does significantly increase plasma PAI-1 levels.

The LDLR gene family consists of several members. Among them are the LDLR, very low-density lipoprotein receptor (VLDLR), apolipoprotein E receptor (apoE-R) and megalin. We demonstrated that next to LRP both LDLR and VLDLR are also not involved in the regulation of plasma PAI-1 levels. Which of the other RAP-sensitive mechanisms regulates plasma PAI-1 levels remains to be elucidated. The RAP-sensitive megalin might be good candidate for the regulation of plasma PAI-1. Megalin is shown to interact with PAI-1 and mediates the endocytosis of PAI-1 for degradation *in vitro*.[6,7] Although PAI-1/LRP interaction does not influence plasma levels, the interaction between PAI-1 and LRP might be of importance locally at the cellular level. This PAI-1/LRP interaction might be of importance in the clearance of proteins of the haemostasis system, such as thrombin. PAI-1 can promote the clearance of thrombin via LRP.[7] Additionally, PAI-1 is a potent chemo-attractant molecule, which activity depends on the interaction with LRP for cell signalling.[8] Altogether, we propose that the interaction between PAI-1 and LRP is of importance on a local level rather than on the plasma level.

In 1986, PAI-1 was firstly described to be linked to insulin resistance.[9] Increased plasma PAI-1 levels and further progression of the increased plasma PAI-1 levels correlate strongly with insulin resistance.[10,11] Improvement of insulin resistance by diet or pharmacologic intervention is correlated with decreased plasma PAI-1 levels.[12,13] Diet-induced obesity and insulin resistance were prevented in PAI-1 deficient mice on a wild-type background.[14] Increased plasma PAI-1 levels are suggested to be the result of increased expression of PAI-1 by adipose tissue and ectopic fat depots.[10] These studies together generate the hypothesis that PAI-1 can cause the development of obesity and insulin resistance. It is not known whether attenuated plasma PAI-1 clearance contributes to the increased plasma PAI-1 levels observed in insulin resistance. In **chapter 2** we demonstrated that increased plasma PAI-1 levels in diet-induced insulin resistant obese mice is not due the decreased plasma PAI-1 clearance. Moreover, we showed that plasma PAI-1 levels followed obesity and insulin resistance in time with delay of weeks suggesting that PAI-1 is not causally related to insulin resistance. Several other studies support our data. The study by Morange *et al* showed that PAI-1 deficient mice develop more adipose tissue.[15] Transgenic mice overexpressing PAI-1 have lower body weight, lower adipose tissue mass and less intraperitoneal fat.[16] A new hypothesis might be proposed that the increased PAI-1 levels observed in the epidemiological studies and animal studies are an epiphenomenon of inflammation in the setting of insulin resistance and obesity.

PAI-1 is an acute phase protein and can be induced by inflammation such as induced by lipopolysaccharide injection.[17] Obesity and insulin resistance are positively correlated with low-grade inflammation. Inhibition of inflammation is demonstrated to decrease plasma PAI-1 levels in subjects with insulin resistance.[13] The pro-inflammatory marker tumor necrosis factor-α (TNF-α) is elevated in obese human subjects and rodents.[18] Obese mice deficient of TNF-α remain insulin sensitive.[19] TNFα is known to induce the expression of PAI-1.[20] Taken altogether, these findings support our hypothesis that increased plasma PAI-1 levels are an epiphenomenon in the setting of insulin resistance and obesity rather than a causal factor. The data from the different PAI-1 deficient mouse models could be explained by the different environment/housing or genetically different inflammatory background. This epiphenomenon of increased plasma PAI-1 levels in the setting of insulin resistance could be of significant clinical importance in regard to cardiovascular events. Subjects with insulin resistance have increased cardiovascular risk at the same amount of atherosclerotic burden compared to subjects without insulin resistance. It is apparent that many studies are still needed to investigate the exact mechanisms underlying the involvement of PAI-1 in the development of insulin resistance and obesity.

Role of LRP in atherosclerosis

The traditional view of the development of atherosclerosis is the accumulation of cholesterol in the vessel wall facilitated by elevated plasma cholesterol levels packed in lipoproteins. These lipoproteins are processed and hydrolysed during the transport before taken up by the liver by the LDLR and LRP or the peripheral tissue by the VLDLR. Alternative pathways for the clearance of the triglyceride (TG)-rich lipoproteins are heparin sulphate proteoglycans (HSPGs) and the scavenger receptor class B type I (SR-BI).[21-23] Lipoprotein lipase (LPL) is the key enzyme responsible for the hydrolysis of TG-rich lipoproteins and is suggested to play a role in the uptake of the TG-rich lipoproteins by the LDLR and LRP *in vitro*.[24] By using the LRP-LDLR-/-VLDLR-/- mouse model (**Chapter 4**), we demonstrated that LPL activity also regulates the hepatic uptake of TG-rich lipoproteins via a pathway different from the lipoprotein receptors pathway. We confirmed that both the HSPG and SR-BI pathways are also involved in the clearance of the TG-rich lipoproteins. Therefore plasma lipoprotein levels not only depend on the activity of the lipoprotein receptors, but also on the activity of LPL and the non specific receptors HSPGs and SR-BI. As a consequence, impaired activity of these pathways can result in the development of hyperlipidemia, and eventually atherosclerosis.[25,26]

Atherosclerosis is also considered as an inflammatory disease of the vascular wall.[27] Macrophages play a central role in the pathogenesis of atherosclerosis. Several lines of evidence suggest that LRP in the macrophages promotes the development of atherosclerosis, since it stimulates foam cell formation.[28-35] However, these are all indirect *in vitro* studies. Therefore, we took the advantage of the unique mouse model of macrophage specific LRP deficiency to investigate the role of LRP in macrophages in the development of atherosclerosis **(Chapter 5)**. We demonstrated that LRP in the macrophages protects against the development of atherosclerosis. The mechanism by which macrophage LRP modulates atherosclerosis is not clear yet. A careful control of the balance between the pro-atherogenic and anti-atherogenic LRP ligands is obviously necessary. As important is a strict regulation of cell migration and remodelling of the extracellular matrix. Therefore, it can be postulated that the LRP in the macrophages plays a central role in both controlling the balance between the pro-atherogenic and anti-atherogenic ligands, and the regulation of the extracellular matrix, since it is a multi-ligand multifunctional receptor.[36-40] This hypothesis is supported by tissue specific LRP deficient mouse models. Hepatic LRP deficiency results in increased plasma apoE-rich lipoproteins. Additionally, independent of the plasma lipoproteins LRP deficiency in the liver and smooth muscle cell (SMC) results in increased atherosclerosis and impaired vascular structure.[40,41] In the absence of LRP, the accumulation of pro-atherogenic ligands was observed and the tight regulation of cell migration and the remodelling of the extracellular matrix obliterated. We demonstrated that LRP deficiency in the macrophage results in increased extracellular collagen matrix. Since the extracellular collagen accumulates

in the atherosclerotic plaque in the absence of LRP in the macrophage, one can propose that LRP deficiency results in a stable plaque. However, the definition of a stable plaque is disputable. One could also propose that LRP in the macrophage controls the regulation of cell migration and remodelling of the extracellular matrix as LRP in the SMC. The extracellular collagen is tightly controlled amongst others by the matrix metalloproteinase (MMP)/tissue inhibitor metalloproteinase (TIMP) system and interferon-γ (IFN- γ).[42,43] MMP-9 is an important representative of the MMP/TIMP system and is a known ligand of LRP.[44] The role of MMP-9 is not unambiguous. We did not observe increase of MMP-9. On the other hand, extracellular collagen is modulated by T cells via IFN- γ. T cells inhibit the production of the extracellular matrix stimulated by INF- γ. We demonstrated that the number of T cells was decreased in the atherosclerotic plaques when LRP is absence in the macrophages. One could also propose that LRP in the macrophages controls the inflammatory process in the development of atherosclerosis. This hypothesis is supported by the observed increased inflammatory markers, such as the monocyte chemo-attractant protein type-1 (MCP-1) and TNF-α, present in the atherosclerotic plaques in macrophage LRP deficient mice.[45] It is also suggested that LRP in the macrophage suppresses the inflammation via the NF-κB pathway.[46] All these evidences are in line with the current conception that atherosclerosis is an inflammatory disease of the vascular wall.

Role of inflammation in atherosclerosis in subjects with MetS

MetS is associated with chronic low-grade systemic inflammation. More and more evidence is accumulating that inflammatory markers are predictive to cardiovascular risks. One of the inflammatory markers associated with MetS is the C-reactive protein (CRP).[47] CRP is shown to be associated with endothelial damage and atherosclerosis.[48] Under physiological conditions endothelial damage is restored by the circulating endothelial progenitor cells (EPC). After incorporation into the endothelial monolayer, the EPC stimulate the proliferation of the neighbouring endothelial cells restoring the damaged endothelium.[49] Circulating EPC are thought to be derived from hemangioblastic cells that reside in the bone marrow. Lower numbers of circulating EPC are observed in subjects with cardiovascular diseases.[50] We demonstrated that elevated inflammation as measured by CRP levels is associated with decreased numbers of circulating EPC in subjects with the MetS (**Chapter 6**). The presence of atherosclerotic lesions as assessed by magnetic resonance imaging (MRI) also correlates with lower number of EPC. Very interesting is the similar observation in other chronic inflammatory diseases such as rheumatoid arthritis.[51] Therefore, one can propose that lower circulating EPC levels are associated with a chronic inflammatory state. The mechanism underlying these observations is not completely clear. Is the increased utilisation of EPC by the damaged endothelium an explanation for the low number of circulating plasma EPC? Or is it the inflammatory state that suppresses the production of EPC? Or is it both? We demonstrated that the number hematopoietic stem cells (HSC) is decreased when

low-grade inflammation is present. We also showed that the lower numbers of EPC and HSC are accompanied by higher levels of inflammatory markers like TNF-α and p-selectin. Therefore, suppressed production might be an explanatory factor. However, the questions remain unsolved since our study was an observational study. To elucidate the underlying mechanism, *in vitro* studies need to be performed on how inflammatory markers can influence the number or the function of the EPC and HSC.

Concluding remarks

It should be realised that multiple factors are involved in the pathophysiology of the metabolic syndrome and its increased risk for cardiovascular diseases. Although intensive research is being performed, much research is still required before therapeutic interventions targeting the metabolic syndrome can be developed.

In this thesis we showed the PAI-1 catabolism is facilitated by a RAP-sensitive mechanism other than LRP, LDLR and VLDLR. The increased plasma PAI-1 levels observed in insulin resistance and obesity is not explained by impaired clearance of PAI-1. The increased plasma PAI-1 levels might be an epiphenomenon of the chronic inflammatory state of insulin resistance or obesity. Furthermore, alternative pathways other than the traditional lipoprotein receptors are involved in the regulation of plasma cholesterol and triglyceride levels. The development of atherosclerosis is multi-factorial in which the balance between the anti- and pro-inflammatory processes plays a central role. Macrophage LRP might be one of the features that control this balance. Inflammation not only promotes to the development of atherosclerosis, but might also be involved in the processes that restore the damaged vascular wall. Insulin resistance and cardiovascular diseases are becoming epidemic in the western world concerning both children and adults. Since increased chronic inflammation is particularly fundamental to this, demanding research on this aspect of metabolic syndrome is very much needed.

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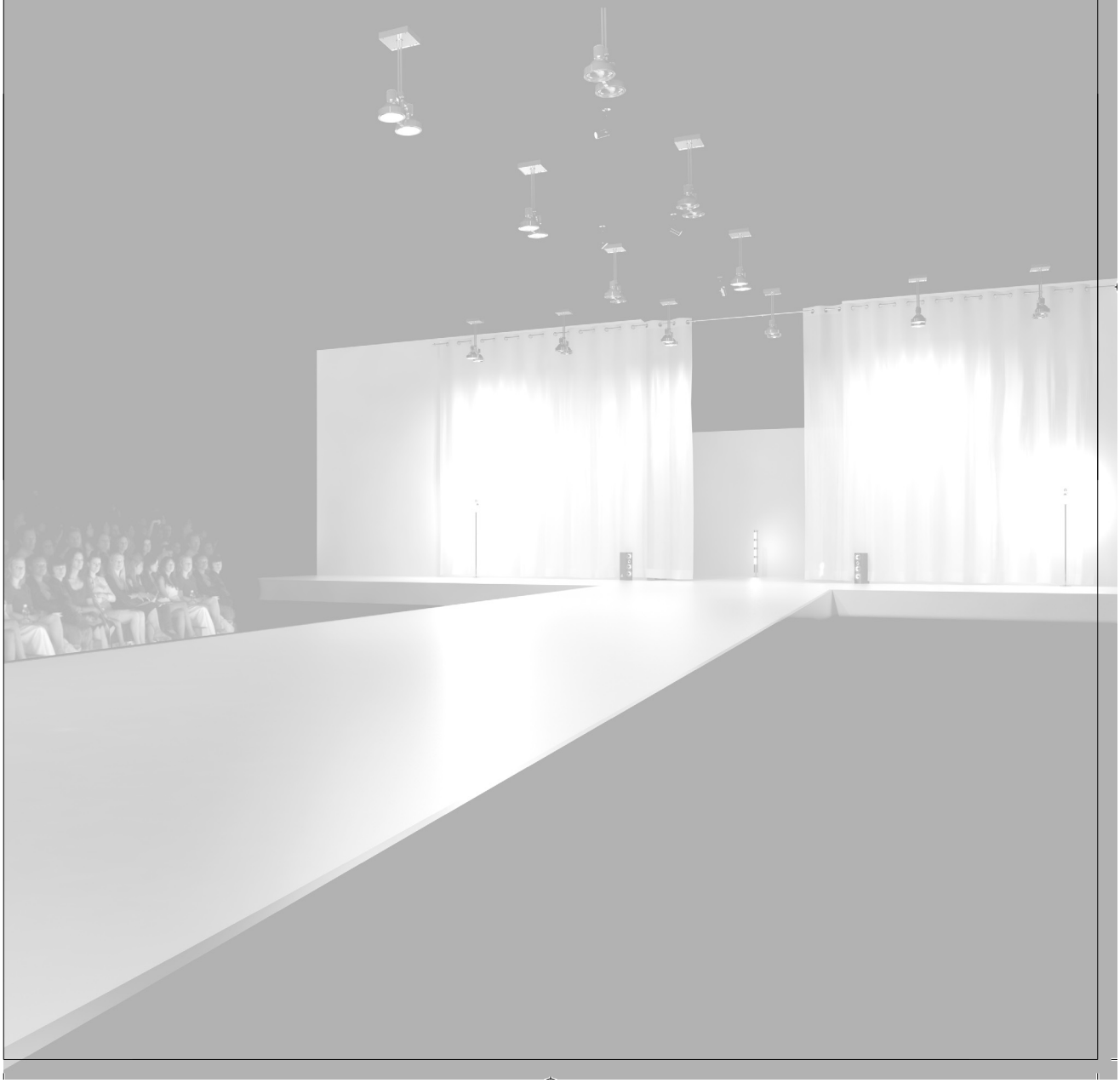
Chapter 8

Summary

Samenvatting



Summary

The metabolic syndrome is an increasing problem in our Western society. It involves multiple aspects like obesity, insulin resistance, dyslipidemia and low-grade chronic inflammation. Subsequently, it has a major impact on the development of atherosclerosis, therefore cardiovascular diseases. The aim of this thesis was to expand our knowledge of the different aspects of the metabolic syndrome at the molecular level, specifically the plasminogen activator inhibitor-1 (PAI-1), insulin resistance, low-density lipoprotein receptor-related protein (LRP) and atherosclerosis. For this purpose, we used different transgenic mouse models and an observational study with human subjects.

Obesity and insulin resistance are key components of the metabolic syndrome. Plasma PAI-1 levels are increased in patient with the metabolic syndrome. The increased plasma PAI-1 levels are suggested to be the result of increased expression in the vascular endothelium, adipose tissue and liver. However, it is not known if the clearance also contributes to the increased plasma PAI-1 levels. **Chapter 2** describes the clearance of plasma PAI-1 in a genetically and a diet-induced insulin resistant mouse models. We showed that increased plasma PAI-1 levels in diet-induced insulin resistant obese mice are not due to decreased plasma PAI-1 clearance. We also demonstrated plasma PAI-1 levels followed obesity and insulin resistance in time. These data suggest that PAI-1 is probably not causally involved in the development of insulin resistance. Increased PAI-1 levels might merely be an epiphenomenon in the setting of insulin resistance and obesity.

PAI-1 interacts with the low-density lipoprotein receptor (LDLR)-related protein (LRP) *in vitro.* A number of studies have shown that LRP can bind, internalise and degrade PAI-1 *in vitro*. However, it is not known whether LRP indeed plays a role in the clearance of plasma PAI-1 *in vivo*. **Chapter 3** addressed the role of hepatic LRP in the regulation of plasma PAI-1 *in vivo*. For this purpose, we studied the clearance of PAI-1 in hepatic LRP deficient mice under different conditions. We showed that hepatic LRP is not involved in the regulation or clearance of plasma PAI-1 levels. Additionally, we demonstrated that receptor-associated protein (RAP)-sensitive mechanism other than the very low density lipoprotein receptor (VLDLR) or the low density lipoprotein receptor (LDLR) is involved in the regulation of plasma PAI-1 levels.
Hepatic LRP deficient mice have elevated fasted plasma cholesterol and triglyceride levels, mainly present as VLDL particles on a LDLR-/-VLDL-/- background. Since VLDL is continuously produced in the liver, VLDL remnants still need to be cleared to maintain a steady state level. **Chapter 4** addressed the whether LPL activity is important for the hepatic clearance of VLDL remnants independent of the three major apoE-recognizing receptors LRP, LDLR and VLDLR. For this purpose, we used mice deficient of LRP, LDLR and VLDLR. We

demonstrated that the clearance of triglyceride (TG)-rich lipoproteins depends on the LPL activity and the non-specific receptors heparin sulfate proteoglycans (HSPG) and scavenger receptor BI (SR-BI) in the absence of the three major lipoprotein receptors LDLR, LRP and VLDLR.

LRP in the liver and SMC is shown to have atheroprotective role. Macrophages play a key role in the development of atherosclerosis next to SMC. Data from several *in vitro* studies suggest a pro-atherogenic of LRP in the macrophage. In **chapter 5** we investigated the role macrophage LRP in the development of atherosclerosis *in vivo*. We demonstrate that, independent of its role in lipoprotein uptake, absence of LRP in macrophages resulted in more advanced atherosclerosis and in lesions that contained more collagen and less CD3+ T cells. In contrast to previous *in vitro* studies, we conclude that macrophage LRP has an atheroprotective potential and may modulate the extracellular matrix in the atherosclerotic lesions.

Patients with the metabolic syndrome have chronic low-grade inflammation and increased risk for cardiovascular diseases. **Chapter 6** describes the influence of low-grade inflammation on the number of endothelial progenitor cells (EPCs) in patients with the metabolic syndrome. EPCs are thought to restore the endothelial layer when damaged. Here we demonstrated that increased low-grade inflammation as reflected by increased C-reactive protein (CRP) levels is accompanied by decreased numbers of EPCs and hemopoeitic stem cells (HSCs) in subjects with the metabolic syndrome. Subjects with atherosclerotic plaques have also lower number of EPCs and HSCs as compared to subjects without atherosclerotic plaques. Therefore, low-grade inflammation is suggested to affect the vascular damage.

Taken together, the studies in this thesis contribute to understanding of the different molecular aspects of the metabolic syndrome, in particular PAI-1, insulin resistance, LRP and atherosclerosis. However, much further research is still needed to understand the metabolic syndrome and the subsequent cardiovascular diseases.

Samenvatting

Het metabool syndroom is een toenemend probleem in onze westerse samenleving. Verschillende aspecten zoals vetzucht, insuline resistentie, dyslipidemie and laag gradig chronische ontsteking. Het gevolg hiervan is dat het metabool syndroom grote impact heeft op de ontwikkeling van atherosclerose (aderverkalking) en daarmee ook hart- en vaatziekten. Het doel van dit proefschrift was de kennis van de verschillende aspecten van het metabool syndroom op het moleculaire niveau te vergroten. In het bijzonder worden de aspecten van plasminogeen activator inhibitor-1 (PAI-1), insuline resistentie, low-density lipoprotein receptor (LDLR)-related protein (LRP) en atherosclerose bestudeerd. Hiervoor hebben we gebruik gemaakt van verschillende transgene muismodellen en een observationele studie bij de mensen.

Overgewicht en insuline resistentie zijn de meest belangrijkste componenten van het metabool syndroom. Plasma PAI-1 concentraties zijn verhoogd in patiënten met het metabool syndroom. Deze verhoogde plasma PAI-1 concentraties van de plasma PAI-1 concentraties wordt beschouwd als het gevolg van toegenomen expressie door het vaatwand, de vetcellen en de lever. Het is echter nog niet bekend of de klaring van PAI-1 ook resulteert in verhoogde plasma PAI-1 concentraties. In **hoofdstuk 2** wordt de klaring van plasma PAI-1 in een genetisch en dieet geïnduceerde insuline resistent muis model bestudeerd. We hebben laten zien dat de toegenomen plasma PAI-1 concentraties in een dieet geïnduceerde insuline resistente obese muismodel niet verklaard kan worden door verminderde klaring van PAI-1. Daarnaast hebben we ook laten zien dat toename van plasma PAI-1 concentraties later ontstaat dan overgewicht en insuline resistentie in de tijd. Deze data suggereren dat PAI-1 waarschijnlijk niet causaal betrokken is bij de ontwikkeling van insuline resistentie. De waargenomen toegenomen PAI-1 concentraties kan mogelijk enkel een epifenomeen zijn in de setting van insulin resistentie en overgewicht.

Uit literatuur van *in vitro* studies is gebleken dat PAI-1 een interactie aangaat met LRP. Een aantal studies hebben laten zien dat LRP PAI-1 kan binden, opnemen en afbreken. Het is echter niet bekend welke rol LRP heeft in de klaring van PAI-1 *in vivo*. **Hoofdstuk 3** beschrijft de rol van de LRP in de lever in de regulatie van plasma PAI-1 *in vivo*. Hiervoor hebben we de klaring van PAI-1 in muizen zonder LRP in verscheidene condities bestudeerd. We hebben laten zien dat LRP in de LRP niet betrokken is bij zowel de regulatie als de klaring van plasma PAI-1. Bovendien laten we zien dat de receptor-associated protein (RAP) sensitieve mechanisme betrokken zijn bij de regulatie van plasma PAI-1. De RAP sensitieve receptoren LDLR en VLDLR zijn hierbij net als LRP niet betrokken.

Muizen met lever LRP deficiëntie hebben een verhoogde nuchter plasma cholesterol en triglyceride concentraties. De cholesterol en triglyceride in deze LRP deficiënte muizen zijn voornamelijk in VLDL partikels aanwezig op een LDLR-/- en VLDLR-/- achtergrond. Aangezien VLDL partikels continu worden geproduceerd door de lever, moeten de afgebroken VLDL deeltjes nog steeds worden geklaard om een steady state niveau te behouden. In **hoofdstuk 4** wordt de activiteit van lipoprotein lipase (LPL) in de klaring van de afgebroken VLDL deeltjes bestudeerd in de afwezigheid van LRP, LDLR en VDLDR, de drie belangrijkste apolipoprotein E herkennende receptoren. Hiervoor hebben we gebruik gemaakt van muizen die deficiënt zijn voor LRP, LDLR en VLDLR. We hebben laten zien dat de klaring van triglyceride rijke lipoproteines afhankelijk is van de activiteit van LPL en van heparin sulfate proteoglycanen (HSPG) en scavenger receptor B1 (SR-BI), de non-specifieke receptoren wanneer LRP, LDLR en VLDLR afwezig zijn.

LRP in de lever en in de gladde spiercellen hebben een beschermende rol in de ontwikkeling van atherosclerose. Macrofagen spelen naast de gladde spiercellen een centrale rol bij de ontwikkeling van atherosclerose. Data van verscheidene *in vitro* studies suggereren dat LRP in de macrofaag juist atherogeen is. In **hoofdstuk 5** hebben we de role van LRP in de ontwikkeling van atherosclerose *in vivo* onderzocht. We hebben laten zien dat de afwezigheid van LRP in de macrofagen juist atherosclerose verergert. Hierbij was het lipoproteine metabolisme onafhankelijk van LRP. De atherosclerotische plaques bevat meer collagen en minder CD3+ T cellen. In tegenstelling tot de in vitro studies, concluderen we dat LRP in de macrofagen een beschermende rol heeft in de ontwikkeling van atherosclerose mogelijk door het moduleren van de extracellulaire matrix in de atherosclerotische plaques.

Patiënten met het metabool syndroom hebben chronisch laaggradige ontsteking en verhoogde risico op hart- en vaatziekten. **Hoofdstuk 6** beschrijft het gevolg van laaggradige ontsteking op het aantal endotheliale voorloper cellen (EPC's) in patiënten met het metabool syndroom. EPC's herstellen het beschadigde endotheel. In dit hoofdstuk laten we zien dat verhoogd laaggradige ontsteking, weerspiegeld door verhoogde C-reactief protein (CRP) concentraties, is geassocieerd met verminderd aantal EPC en hemopoetischce stam cellen (HSC's) in mensen met het metabool syndroom. Mensen met atherosclerose hebben ook een lager aantal EPC's en HSC's in vergelijking met mensen zonder atherosclerose. Dit suggereert dat laaggradige ontsteking een invloed heeft op de beschadiging van het endotheel.

Concluderend, de studies beschreven in dit proefschrift leiden tot meer het begrip van de verschillende aspecten van het metabool syndroom, m.n. PAI-1, insuline resistentie, LRP en atherosclerose. Veel onderzoek is echter nog noodzakelijk om het metabool syndroom te begrijpen en daarmee haar hart- en vaatziekten.

List of Abbreviations

ApoCI	apolipoprotein CI
ApoCII	apolipoprotein CII
ApoCIII	apolipoprotein CIII
apoE	apolipoprotein E
ApoE-R2	apolipoprotein E receptor 2
CRP	C-reactive protein
CVD	cardiovascular diseases
DM II	Type II diabetes mellitus
EGF	epidermal growth factor
EGIR	European Study Group of Insulin Resistance
EPC	endothelial progenitor cells
FFA	free fatty acids
HL	hepatic lipase
ICAM	intracellular cell adhesion molecule-1
IDF	International Diabetes Federation
IL-10	Interleukin-10
IL-6	Interleukin-6
IFN-γ	interferon-γ
LDL	low-density lipoprotein
LDLR	low-density lipoprotein receptor
LPL	lipoprotein lipase
LRP	low-density lipoprotein receptor-related protein
MCP-1	chemoattractant protein-1
M-CSF	macrophage colony-stimulating factor
MetS	Metabolic syndrome
NCEP ATP III	National Cholesterol Education Program Adult Treatment Panel III
NF-κB	nuclear factor κB
PAI-1	plasminogen activator inhibitor-1
PDGF	platelet-derived growth factor
PDGF-BB	platelet-derived growth factor isoform BB
RAP	receptor-associated protein
SAA	serum amyloid A
TG	triglyceride
TNF-α	tumor necrosis factor-α
uPA	urokinase plasminogen activator
uPAR	urokinase plasminogen activator receptor
VCAM-1	vascular cell adhesion molecule-1
VLDL	very low-density lipoprotein
VLDLR	very low-density lipoprotein receptor
VSMC	vascular smooth muscle cells
WHO	World Health Organisation
WT	wild-type

List of Publications

Full papers

1. de Leeuw van Weenen JE, Hu L, Jansen-van Zelm K, de Vries, MG, Tamsma JT, Romijn JA, Pijl H: Four weeks high fat feeding induces insulin resistance without affecting dopamine release or gene expression patterns in the hypothalamus of C57Bl6 mice. **Brain Res. 2008**, *in press*

2. Hu L, Van der Hoogt CC, Espirito Santo SM, Out R, Kypreos KE, Van Vlijmen BJ, Van Berkel TJ, Romijn JA, Havekes LM, Willems van Dijk K, Rensen PC: The hepatic uptake of VLDL in absence of the three major apoE-recognizing receptors is regulated by LPL activity and involves heparan sulfate proteoglycans and scavenger receptor BI. **J Lipid Res**. **2008**, 49(7):1553-61

3. Hu L, Bovenschen N, Havekes LM, van Vlijmen BJ, Tamsma JT: Plasma plasminogen activator inhibitor-1 level is not regulated by the hepatic low-density lipoprotein receptor-related protein. **J Thromb Haemost. 2007**, 5(11):2301-4.

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6. Bovenschen N, Mertens K, Hu L, Havekes LM, van Vlijmen BJ: LDL receptor cooperates with LDL receptor-related protein in regulating plasma levels of coagulation factor VIII in vivo. **Blood. 2005**, 106(3):906-12.

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8. Hu L, Berbée JFP, van Vlijmen BJM, Havekes LM, Tamsma JT: The plasma PAI-1 levels is not affected by the clearance in insulin resistant mice. *Submitted for publication*

9. Alizadeh Dehnavi R, Hu L, de Boer HC, van Zonneveld AJ, de Roos A, van Pelt J, Putter H, Romijn JA, Rabelink TJ, Tamsma JT: Decreased circulating endothelial progenitor cell counts accompany elevated CRP levels in subjects with the metabolic syndrome without diabetes or overt cardiovascular disease. *Submitted for publication*

10. Alizadeh Dehnavi R, de Boer HC, van der Kraan J, Hu L, van Zonneveld AJ[2], de Roos A, van Pelt J, Putter H, Romijn JA, Rabelink TJ, Tamsma JT: Effect of rosiglitazone on circulating endothelial progenitor cells in non-diabetic patients with metabolic syndrome and inflammation. *Submitted for publication*

11. de Vries- van der Weij J, de Haan W, Hu L, Kuif M, Oei HLDW, van der Hoorn J. Havekes LM, Princen HMG, Romijn JA, Smit JWA, Rensen PCN: Bexarotene induces dyslipidemia by increased VLDL production and CETP-mediated reduction of HDL. *Submitted for publication*

Abstracts

Hu L, Boesten LSM, May P, Herz J, Bovenschen N, Huisman MV, Berbee JFP, Havekes LM, Van Vlijmen BJM, Tamsma JT: Macrophage low-density lipoprotein receptor-related protein deficiency enhances atherosclerosis. Atherosclerosis 2006, 7(3):331

Hu L, Boesten L, Huisman M, Meinders E, Havekes L, van Vlijmen B, Tamsma J: Macrophage low-density lipoprotein receptor-related protein deficiency enhances atherosclerosis. Journal of vascular research 2005, 42(suppl 2):55

Curriculum Vitae

Lihui Hu werd geboren op 21 juni 1976 te Yu Hu, Zhe Jiang province, China. In 1985 is zij verhuisd naar Nederland. Na het behalen van haar VWO diploma in 1995 aan het Titus Brandsma Lyceum te Oss, studeerde ze Biomedische Wetenschappen en Geneeskunde aan de Universiteit Leiden. De propedeutische examens voor Biomedische Wetenschappen en Geneeskunde werden respectievelijk in augustus 1996 en augustus 2000 gehaald. In het kader van haar hoofdvakstage van Biomedische Wetenschappen werd onderzoek verricht bij 'The Department of Molecular and Cellular Physiology, Yale University' onder leiding van dr.B.M. Schmitt and prof. Dr. W.F. Boron. In het kader van haar keuze co-schap voor Geneeskunde heeft zij gewerkt bij 'Hyperbaric Medical Unit' van 'The Townsville Hospital' Townsville, Australië onder leiding van dr. D. Griffiths. De doctoraal examens Biomedische Wetenschappen en Geneeskunde werden respectievelijk in maart 2004 en januari 2004 met goed gevolg afgelegd.

Van april 2004 tot en met augustus 2007 was zij als arts-onderzoeker werkzaam bij de afdeling BioMedical Research van TNO-Kwaliteit van Leven en de afdeling Interne Geneeskunde van het Leids Universitair Medisch Centrum te Leiden. Tijdens deze periode werd het in dit proefschrift beschreven onderzoek uitgevoerd onder leiding van Prof. Dr. Ir. L.M. Havekes, Dr. J.T. Tamsma en Dr. B.J.M. van Vlijmen. Zij werd mede gefinancierd haar NWO Mozaïek beurs welke verkregen is in 2006. Zij won de Young Investigator Award 2006 van de 12^{e} LVM Scientic Meeting en een Travelar Grant 2006 van de XIV International Symposium on Atherosclerosis.

Sinds september 2007 is zij in opleiding tot internist in het HAGA ziekenhuis te Den Haag en het Leids Universitair Medische Centrum.